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This study on prevention of human mammary carcinogenesis utilized *in vitro* models from non-cancerous human breast. Carcinogenesis was initiated by the environmental carcinogen BP or the HER-2/neu oncogene. Preneoplastic transformation was evaluated by determining expression of select biomarkers. Ras p21-GTP binding, estradiol metabolism, DNA synthesis, cell cycle regulatory gene expression, apoptosis and aberrant hyperproliferation represented the biomarkers. The preventive efficacy of naturally-occurring compounds I3C, EPA, EGCG and GEN was evaluated by determining the down-regulation of the perturbed biomarkers. In the explant culture model, BP increased Ras p21-GTP binding and DNA synthesis, and decreased C2/C16α-hydroxylation ratio of estradiol. Treatment of BP-initiated cultures with EPA or I3C resulted in decreased Ras p21-GTP binding and DNA synthesis, and increased C2/C16α-hydroxylation ratio. In the cell culture model, BP or HER-2/neu increased S and/or G_2+M phases of the cell cycle and immunoreactivity to positive growth regulators. This aberrant hyperproliferation was accompanied by inhibition of apoptosis and altered immunoreactivity to apoptosis-associated gene products. Treatment of initiated cells with I3C, EGCG or GEN resulted in differential down-regulation of perturbed biomarkers. This study provides a clinically relevant experimental approach to evaluate prevention of human mammary carcinogenesis.

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Nitin T. Telang, 07/30/96

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ABSTRACT

This study on prevention of human mammary carcinogenesis utilized *in vitro* models from non-cancerous human breast. Carcinogenesis was initiated by the environmental carcinogen BP or the HER-2/neu oncogene. Preneoplastic transformation was evaluated by determining expression of select biomarkers. Ras p21-GTP binding, estradiol metabolism, DNA synthesis, cell cycle regulatory gene expression, apoptosis and aberrant hyperproliferation represented the biomarkers. The preventive efficacy of naturally-occurring compounds I3C, EPA, EGCG and GEN was evaluated by determining the down-regulation of the perturbed biomarkers. In the explant culture model, BP increased Ras p21-GTP binding and DNA synthesis, and decreased C2/C16α-hydroxylation ratio of estradiol. Treatment of BP-initiated cultures with EPA or I3C resulted in decreased Ras p21-GTP binding and DNA synthesis, and increased C2/C16α-hydroxylation ratio. In the cell culture model, BP or HER-2/neu increased S and/or G_2+M phases of the cell cycle and immunoreactivity to positive growth regulators. This aberrant hyperproliferation was accompanied by inhibition of apoptosis and altered immunoreactivity to apoptosis-associated gene products. Treatment of initiated cells with I3C, EGCG or GEN resulted in differential down-regulation of perturbed biomarkers. This study provides a clinically relevant experimental approach to evaluate prevention of human mammary carcinogenesis.

I. INTRODUCTION:

This final progress report summarizes the experiments performed and results obtained during a two year funding of Department of the Army grant #DAMD17-94-J-4208 for the period of July 1, 1994 through June 30, 1996. The experiments utilized the human mammary terminal duct lobular unit (TDLU) explant culture and the human mammary epithelial 184-B5 cell culture models to examine whether i.) exposure to the environmental carcinogen Benzo(α)pyrene (BP) or overexpression of the HER-2/neu oncogene induces perturbation in select biochemical and cellular surrogate endpoint biomarkers (SEPB) and ii.) treatment of initiated TDLU or 184-B5 models with select naturally-occurring dietary agents results in down-regulation of perturbed SEPB. During the first year of funding (July 1, 1994 through June 30, 1995) research was focused on the TDLU explant culture model and on optimizing the newly identified SEPB for the 184-B5 cell culture model. During the second year of funding (July 1, 1995 through June 30, 1996) the cell culture model and SEPB were utilized to evaluate the preventive efficacy of the naturally-occurring dietary agents.

A. Nature of the problem: Breast cancer is one of the prevalent causes of mortality in women. The American Cancer society has estimated a 31% incidence (184,300 new breast cancer cases) and a 17% death rate (44,300 cancer related deaths) in 1996. (1). The multi-stage process of mammary carcinogenesis involves early-occurring events of initiation and promotion of preneoplastic transformation, and late-occurring events that influence expression of the tumorigenic and invasive phenotype (2-4). In this carcinogenic process, interactive influences of genetic, environmental, endocrine and dietary factors represent major determinants for predisposition and/or progression of the disease (1, 3-5).

Laboratory studies on animal models have provided important leads for etiology, pathogenesis and prevention of human mammary carcinogenesis (5-12). The clinical relevance of the laboratory evidence is dependent on extrapolation, and therefore, is equivocal.

Identification and validation of a spectrum of surrogate endpoint biomarkers specific for cancer risk, early detection and efficacious primary and/or secondary prevention, therefore, remains a high priority area of research (2-5). Investigations on appropriate human tissue-derived models should provide a facile approach to reduce the need for extrapolation and to facilitate mechanism-driven translational research (3-5, 13-15, 29, 32, 33).

B. Background of previous work: Experiments with *in vitro* models for rodent mammary carcinogenesis have demonstrated that prototypic initiators such as chemical carcinogens, oncogenes and transforming retrovirus induce preneoplastic and tumorigenic changes in the non-cancerous target tissue. (6, 18, 35-44). In addition, a spectrum of molecular, biochemical, endocrine and cellular surrogate endpoint biomarkers has been

identified and validated that represent quantitative parameters to evaluate induction and modulation of preneoplastic transformation (6, 18, 35, 36). In an effort to establish a clinical relevance for *in vitro* models and surrogate endpoint biomarkers, recent experiments have utilized explant and cell culture models established from non-cancerous human mammary tissue (2, 6, 13-15, 18-22). Taken together, the investigations on rodent and human mammary tissues demonstrate that same surrogate endpoint biomarkers represent useful quantitative endpoints for human mammary carcinogenesis.

II. PURPOSE OF THE PRESENT WORK:

This study was designed to establish clinical relevance of the newly identified SEPB as quantitative parameters for effective chemoprevention of human breast carcinogenesis. The completed experiments have utilized the human mammary TDLU-derived explant culture system and human mammary epithelial 184-B5 cell culture system established from a human reduction mammoplasty specimen (26, 34)to examine whether i.) exposure to the chemical carcinogen BP or transfection with the oncogene HER-2/neu results in perturbation of select biochemical and cellular surrogate endpoint biomarkers that are specific for preneoplastic transformation and ii.) treatment of initiated target tissue/cell with naturally-occurring tumor inhibitors eicosapentaenoic acid (EPA), indole-3-carbinol (I3C), β-carotene (β-C), (-)epigallocatechin gallate (EGCG) and genistein (GEN) effectively down-regulate the perturbed biomarkers. These studies have utilized previously optimized SEPB assays such as replicative DNA synthesis, Ras p21-GTP binding and metabolism of estradiol via C2- and C16α-hydroxylation pathways. In addition, recently completed experiments on the 184-B5 cell culture system have utilized newly identified SEPB assays such as cell cycle regulatory gene expression, cellular apoptosis and immunoreactivity to proliferation specific, apoptosis specific and differentiation specific gene products. The selection of naturally-occurring tumor inhibitors to be used in the present study was based on their documented biological effects on mammary tumors *in vivo* or on tumor-derived cell lines *in vitro* (7-12, 15-17, 19, 47, 49). This comparative approach on human TDLU and 184-B5 systems has provided strong evidence that developed SEPB assays represent useful quantitative parameters to evaluate preventive efficacy of naturally-occurring dietary compounds.

III. METHODS OF APPROACH:

The techniques used for TDLU explant cultures and 184-B5 cell cultures are essentially similar to those reported in earlier studies (6, 13, 14, 22, 26 31, 34)). The cultures were maintained in chemically-defined, serum-free DME/F12 or KBM/MEM media supplemented with requisite hormones and growth factors depending upon specific experiments. The various molecular, biochemical, endocrine and cellular surrogate endpoint biomarkers representing the quantitative parameters are presented in AT-1. The biomarker assays and requisite methodology involved are published (6, 10, 13-15, 18, 19, 22, 30, 31, 35-38, 40-45, 48, 49). **In addition, newly optimized assays for cell cycle progression, cellular apoptosis and expression of cell cycle regulatory gene products**

are utilized to examine the protective effects of I3C, EGCG and GEN against BP and/or HER-2/neu initiated carcinogenesis.

The optimum concentrations for individual chemopreventive test compounds were identified by initial dose response experiments. These dose response studies comprised of master control groups, solvent control groups and experimental groups that were treated with at least four serial dilutions of the stock solution of EPA, I3C, β-C, EGCG, and GEN. The stock solutions were made at the maximal solubility of each compound. The maximally non-toxic concentrations identified from 7 day growth assays were used for the biomarker modulation experiments. Typically, one experiment comprised of at least six independent determinations, and the experiment was replicated at least three times. The statistical significance of the differences between the treatment groups was analyzed by combining the data from three independent experiments (cumulative n=18) and using paired t test with analysis of variance (ANOVA) where appropriate.

IV. PROGRESS REPORT:

The experiments completed during the first year (July 1, 1994 through June 30, 1995) utilized the TDLU explant culture system to examine whether the environmental carcinogen BP induces increased expression of such SEPB as Ras -mediated signal transduction and estradiol metabolism, and whether treatment of BP-initiated TDLU explants with the naturally-occurring tumor inhibitors EPA and I3C down-regulate the perturbed SEPB. In addition, preliminary experiments were conducted utilizing the 184-B5 cell culture system to identify the growth pattern of parental 184-B5, BP-initiated 184-B5/BP, and HER-2/neu-initiated 184-B5/HER cells, and optimize the fluorescence-assisted sell sorting (FACS) assay for cell cycle analysis and for cellular apoptosis.

The experiments conducted during the second year (July 1, 1995 through June 30, 1996) of funding were focused on applying the newly identified SEPB assays to examine the chemopreventive efficacy of naturally-occurring tumor inhibitors I3C, EGCG and GEN on 184-B5/BP and 184-B5/HER cells. The SEPB included carcinogen-DNA adduct formation, estradiol metabolism, cell cycle regulatory gene expression, aberrant hyperproliferation and cellular apoptosis.

This report summarizes the results of all the experiments conducted, and documents the current status and disposition of all the projects during the two year funding of the proposal.

A. EXPERIMENTS ON HUMAN MAMMARY TDLU EXPLANT CULTURE SYSTEM:

These experiments were designed to examine the responsiveness of the non-cancerous target tissue to the environmental chemical carcinogen BP and to evaluate the ability of naturally-occurring tumor inhibitors EPA and I3C to modulate the process of carcinogenesis.

1. **Effect of BP:** These experiments conducted on the TDLU explant culture system examined the acute, direct effects of the ubiquitous environmental carcinogen BP. The Ras p21-GTP binding and 16α-hydroxyestrone (16α-OHE_1) formation represented the SEPB. **Specimens from 8 premenopausal patients undergoing surgery for infiltrating ductal carcinoma (IFDC) were used to prepare the TDLU explant cultures. At least six TDLU explants per patient per treatment group were used for determination of Ras p21-GTP binding activity. A similar number of TDLU were used per patient per treatment group to determine the 16α-OHE_1 formation. These experiments were replicated three times using additional TDLU from the same patients.**

The methodology for Ras p21-GTP binding was similar to that previously published.(6,36,42-45). Briefly, total cellular extract was reacted with [α^{32}p]GTP, G-binding proteins were photo affinity labeled by UV at 260nM, and were separated on a 12.5% SDS-PAGE. The authenticity of Ras p21 was confirmed by immunoprecipitation using Ras specific antibody. Ras p21-GTP bound ^{32}P radioactivity was determined by liquid scintillation counting.

For the 16α-OHE_1 formation assay, TDLU explants were incubated for 48hr. with specifically labeled [C16α-^3H] E_2. The culture medium was processed for determination of ^3H_2O according to the method previously published (13,14,43,44,48,49). The amount of ^3H_2O formed represented an indirect measurement of stoichiometric conversion of E_2 via the C16α-hydroxylation pathway to 16α-OHE_1. Based on the specific activity of E_2, amount of 16α-OHE_1 formed, was calculated.

The explant cultures were maintained in a chemically defined, serum-free medium following published procedures (6, 13, 14, 41, 42, 44, 45). Following a 24 hour exposure to predetermined non-toxic dose of 10μg/ml(≈ 39μM) BP, the relative extent of Ras p21-GTP binding and of 16α-OHE_1 formation was determined. BP treatment induced a fourfold increase (P=0.001) in Ras p21-GTP binding, and a sixfold increase (P=0.001) in 16α-OHE_1 formation relative to that observed in the DMSO treated solvent controls (AF-1a, b). Consistent with these observations, our earlier studies on the *in vitro* models from murine mammary tissue have shown up-regulation in the same SEPB (18, 35, 37, 38, 40-45, 48, 49). Taken together, these data provide evidence for the clinical relevance of the two surrogate endpoint biomarkers. **The data generated from a comparative study on murine and human mammary explant cultures has been published (Telang et al Proc. Int. Cancer Congress pp. 1285-1290, 1994, see appended material).**

2. **Effect of EPA and I3C on BP-induced carcinogenesis:** The experiment designed to examine the modulatory influence of naturally-occurring tumor inhibitors EPA and I3C on BP-induced carcinogenesis, utilized aberrant hyperproliferation (^3H-thymidine uptake) and estradiol (E_2) metabolism (C2/C16α-hydroxylation ratio) as the SEPB. **The two SEPB represent a part of the spectrum of molecular,**

biochemical, metabolic and cellular quantitative parameters for the extent of preneoplastic alterations in vitro that precede the development of tumors in vivo. (2,4,6,36). The doses of BP, EPA and I3C used in these experiments are similar to those reported in other studies (2,6,20,42,44). Furthermore, in response to these treatments, a measurable modulation in SEPB is detected. It is also noteworthy that the extent of biomarker expression in the solvent control group (0.1% DMSO) was comparable to that observed in untreated master controls, and therefore represents the constitute level of biomarker expression. This level is used as a baseline against which to compare the agent-induced perturbation. TDLU explants treated with 0.1% DMSO (solvent controls), 10μg/ml BP (carcinogen controls) and 10μg/ml BP + 5μg/ml EPA or 10μg/ml BP + 5μg/ml I3C (experimentals) represented the four treatment groups.

To measure aberrant hyperproliferation, the cultures were pulse labeled with 5μci/ml ^{3}H-thymidine for the last 24 hours prior to harvest at the fourteen day timepoint, and thymidine uptake into cellular DNA was determined by trichloroacetic acid (TCA) percipitable ^{3}H-radioactivity in the tissue homogenates (38, 42, 44). BP treatment induced a 104% increase (P=0.001) relative to that seen in the DMSO group. In BP+EPA and BP+I3C groups the extent of thymidine uptake was 40% and 47% lower (P=0.001) than that seen in BP treated controls (AF-2a). These data demonstrate that BP induces aberrant hyperproliferation in TDLU, which is effectively down-regulated by EPA and I3C.

To determine the status of E_2 metabolism the cultures treated with DMSO, BP, BP+EPA and BP+I3C were incubated with [^{3}H-C2] E_2 or [^{3}H-C16α] E_2 for the last 48 hours of fourteen day duration, and C2- and C16α-hydroxylation was measured using the radiometric assay (13, 15, 38, 40, 48, 49). The data expressed as C2/C16α-hydroxylation ratio (AF-2b) showed that BP treatment resulted in about 80% decrease (P<0.0001) in the ratio relative to that seen in DMSO treated cultures. Furthermore, the BP-induced decrease in the ratio was almost completely abrogated in the presence of EPA or I3C.

In a recently completed study the TDLU explant culture and the 184-B5 cell culture systems have been utilized to examine the role of E_2 metabolism in chemical carcinogen-induced aberrant proliferation and in the prevention efficacy of I3C (Telang et al Environ. Health Perp. 1997, in press). Treatment of TDLU and 184-B5 cells with the prototypic rodent carcinogens DMBA and BP (2,12,20,27,46) resulted in an increase in 16∝-OHE$_1$ formation with concomittant decrease in 2-OHE$_1$ formation. This alteration in E_2 metabolism expressed as C2/C16∝ ratio demonstrated a 27.4% and 92.5% decrease in TDLU treated with DMBA or BP respectively. Similarly, in the 184-B5 cells the two carcinogens induced 27.6% and 91.0% decrease in the C2/C16∝ ratio respectively.

In the TDLU system cotreatment with 39μM BP+50μM I3C decreased aberrant proliferation by about 43%(p=0.001) and increased C2/C16∝ ration by about 12 fold (p=0.005) relative to that observed in TDLU treated with BP alone. In the 184-B5 cell culture system cotreatment with BP+I3C resulted in about 51% (p=0.001)

decrease in aberrant hyperproliferation, a 219% increase (p=0.005) in cellular apoptosis and a 333% increase (p=0.001) in C2/C16∝ ratio.

The data generated form this comparative study on the human mammary explant and cell culture systems demonstrates that i.) human target tissue is susceptible to carcinogenesis similar to that reported previously (2,20,27,28,45), ii.) treatment of carcinogen-initiated target tissue with I3C offers protection against the carcinogenic insult and iii.) aberrant proliferation, cellular apoptosis and E_2 metabolism represent useful SEPB for mammary carcinogenesis and prevention.

The experiments using the TDLU explant culture system as an in vitro model constitute substantial portions of published and/or submitted manuscripts (see sections VI and VIII and appended material).

The experiments utilizing other test compounds such as β-C, EGCG and GEN have not been very instructive in the TDLU system. β-C and GEN showed limited solubility in DMSO or ethanol and therefore it was not possible to achieve maximally effective non-toxic concentrations in the aqueous culture medium. EGCG was substantially less effective than were EPA and I3C in down-regulating the perturbed SEPB. In this context it needs to be emphasized that efficacy of prevention in the TDLU explant culture system may also be dependent upon the effective transport of the compounds through the stromal component to reach the target epithelial component.

In conclusion, our experiments using the TDLU explant culture system have demonstrated that i.) acute direct exposure to BP results in up-regulation of Ras-mediated signal transduction and increased 16α-OHE_1 formation, and ii.) the naturally-occurring tumor inhibitors EPA and I3C effectively down-regulate the perturbed biomarkers. The cellular heterogeneity due to the presence of adipocytic and fibroblastic stromal components, intrinsic to the model, however, precludes mechanistic studies designed to examine the direct response of transformation sensitive target epithelial cell.

B. EXPERIMENTS ON HUMAN MAMMARY EPITHELIAL 184-B5 CELLS:

In an effort to overcome the technical limitations due to cellular heterogeneity present in the TDLU explant culture model, the reduction mammoplasty-derived 184-B5 mammary epithelial cell culture model was utilized. The experiments were designed to i.) characterize the 184-B5 and 184-B5/HER cells with regard to cell cycle progression, immunoreactivity to cell cycle regulatory gene products, and cellular apoptosis, ii.) examine the biochemical and immunocytochemical alteration in cells initiated for transformation with BP (184-B5/BP) and those with HER-2/neu (184-B5/HER) and iii.) examine the modulatory influence of naturally-occurring tumor inhibitors I3C, EGCG and GEN on cellular transformation induced by BP or HER-2/neu.

1. **Carcinogen-DNA adduct formation in 184-B5 cells:** The ability of chemical carcinogens to form promutagenic DNA adducts has been considered as an early-occurring event in the carcinogenic process and as such, represents a SEPB for genotoxic

DNA damage (27, 28, 39). The experiment presented in AT-2 utilized the [^{32}P] post labeling assay (38, 46) and measured purine nucleotide adducts in 184-B5 cells treated for 24 hours with 39μM DMBA or BP. The untreated 184-B5 cells and those treated with 0.1% DMSO represented the master control and solvent control groups respectively. The metabolism-dependent procarcinogens DMBA and BP produced purine nucleotide adducts, identified as diole epoxide adducts of d-guanine and d-adenine. These data indicate that 184-B5 cells metabolize DMBA or BP and are therefore susceptible to electrophilic DNA damage. Ongoing experimetns are designed to examine whether the treatment of 184-B5/BP with I3C, EGCG or GEN modulate the susceptibility of cells to DNA damage.

2. **Cell proliferation kinetics of 184-B5 and 184-B5/HER cells:** The proliferative kinetics of the parental cell line 184-B5 and HER-2/neu oncogene-initiated 184-B5/HER cell lines was evaluated by determining population doubling time (PDT), saturation density and cell cycle progression. The newly optimized fluorescence-assisted cell sorting (FACS) assay was used for the cell cycle analysis (22, 30, 31). The 184-B5 cells exhibited a PDT of 32.8±1.6 hours and about a twenty-fourfold increase at day 9 post seeding. In contrast, the 184-B5/HER cells exhibit a 34% decrease in PDT and a thirty-eightfold increase at day 9 post seeding as determined by the saturation density data. The cell cycle analysis revealed an increased S phase fraction in 184-B5/HER cells relative to that detected in 184-B5 cells (AT-3). These results indicate that persistent expression of HER-2/neu oncogene may be responsible for the observed aberrant hyperproliferation in 184-B5 cells. Comparative cell cycle analysis of parental 184-B5 and initiated 184-B5/BP or 184-B5/HER cells presented in AT-4 demonstrates increased S phase fraction in cells initiated with BP or HER-2/neu relative to that observed in parental 184-B5 cells. In this context, it is noteworthy that BP as well as HER-2/neu are associated with human carcinogenesis (27-29, 32) and that exposure of non-tumorigenic cells to these agents as well as to other oncogenes confers tumorigenic transformation (2, 6, 20, 21, 26, 34, 35, 39, 40).

3. **Status of cell cycle regulatory gene expression in 184-B5 and 184-B5/HER cells:** The constitutive levels of selected proliferation specific genes such as PCNA, cyclin D_1, cdk 4,6, and 7, and differentiation specific genes EGFR and CAM 5.2-ck was determined by measuring the alteration in cellular immunoreactivity to the specific gene products. **Commercially available antibodies specific for these gene products were used. The antibodies were labeled with FITC whenever necessary following manufacturer's instructions. The extent of cellular immunoreactivity was quantified by determining arbitrary fluorescence unit values that were corrected for specific fluorescence after substracting the non specific fluorescence obtained from staining with FITC labeled IGg(AT-5).**

The data on 184-B5 and 185-B5/HER cells comparing the constitutive levels of select proliferation specific, apoptosis specific and differentiation specific gene products revealed a trend toward higher immunoreactivity to such proliferation specific gene products as cyclin D1 and cdk-7, but not to PCNA and p16. The reason for the lack

of correlation for all the proliferation specific gene products is unclear at present. The data obtained from experiments involving cell proliferative kinetics and cell cycle regulatory gene expression suggests that overexpression of HER-2/neu oncogene induces aberrant hyperproliferation and up-regulation of proliferation specific cell cycle regulatory gene products.

4. **Cellular apoptosis in 184-B5, 184-B5/BP and 184-B5/HER cells:** Apoptosis in concert with proliferation plays an important role in regulation of cellular homeostasis (23-25). The proliferatively active, 1og phase cultures of 184-B5 and 184-B5/HER cells did not exhibit cellular apoptosis as measured by the intensity of Sub G_0 (apoptotic) peak in FACS analysis of propidium iodide stained cell suspension (data not shown). In contrast, quiescent, contact inhibited (confluent) cultures exhibited distinct Sub G_0 peak (AT-6), and nuclear fragmentation was observed by apoptag specific or propidium iodide localized cellular epifluorescence (AF-3a, b). The incidence of apoptotic cells was found to decrease by 88.3% in 184-B5/BP cells (P=0.0001), and by 64.8% in 184-B5/HER cells (P=0.001) respectively, relative to that observed in parental 184-B5 cells (AT-6). **From the data generated on constitutive levels of cellular apoptosis it is clear that this process is down-regulated in response to initiation either by BP or by HER-2/neu. In the 184-B5/HER system decreased, apoptosis is correlated with decreased immunoreactivity to the anti-apoptotic Bcl-2 gene product. (23-25). However, the lack of inhibition of other apoptosis specific gene products such as p53 and Apo-1/fas can not be explained at present. Confirmatory experiments are in progress to establish a correlation between altered immunoreactivity to apoptosis specific gene products and altered incidence of cellular apoptosis. These experiments may provide leads to identify possible mechanisms for modulation of apoptosis.**

5. **Modulation of carcinogenesis by naturally-occurring tumor inhibitors:** To examine whether the process of carcinogenesis induced by BP or HER-2/neu is modulated by naturally-occurring agents that are known to inhibit rodent organ site cancer development (5, 7-12, 19, 33, 42, 44, 47, 49), we selected TDLU explant cultures or 184-B5 cell cultures initiated for carcinogenesis by BP and 184-B5/HER cells that express HER oncogene as the experimental systems. The ability of naturally-occurring compounds I3C, EGCG and GEN to modulate carcinogenesis was evaluated by determining the alterations in E_2 metabolism, aberrant hyperproliferation and cellular apoptosis.

Initial dose response experiments were conducted on 184-B5, 184-B5/BP and 184-B5/HER cells to identify maximally non-toxic concentrations and IC50 for cytostatic concentrations of the naturally-occurring tumor inhibitors I3C, EGCG and GEN. **For the dose response studies a 7-day growth assay was performed. Initially, $1.0x10^5$ cells were plated per T-25 flask. After a 24-hr attachment period, the cultures were exposed continuously to I3C, EGCG or GEN for subsequent 7 days. At the end of this treatment schedule the cultures were typsinized and viable (trypan blue unstained) cell count was determined. The dose ranges examined for each of the test compound were as follows: I3C: 10, 50, 75 and 100μM, 0.1% DMSO (solvent**

control). EGCG:0.22, 2.2, 4.4, 11.0 and 22μM (EGCG was dissolved directly in the culture medium); GEN: 1.0, 2.5, 5.0, and 10μM and 0.1% DMSO as the solvent control. All the three test compounds exhibited a dose-associated growth inhibition in 184-B5 as well as 184-B5/HER cells. In 184-B5 cells I3C treatment resulted in 40%, 75%, 95% and 98% growth inhibition relative to that observed in the solvent treated cultures. EGCG treatment induced 0.2%, 62%, 95%, 96% and 97%, while GEN induced 10%, 57%, 93%, 96% and 97% growth inhibition.

A comparison of the extent of growth inhibition by the test compounds in 184-B5 and 184-B5/HER cells revealed transformation associated, distinct responses to the test compounds. Thus, treatment with 100μM I3C showed a 98% inhibition of growth of 184-B5 cells and 37.8% inhibition of growth of 184-B5/HER cells. Similarly, 22μM EGCG exhibited a 97% growth inhibition of 184-B5 cells and 53.9% growth inhibition of 184-B5/HER cells. Treatment with 10μM GEN resulted in a 97% inhibition in 184-B5 cells, and a 53.2% inhibition in 184-B5/HER cells. Because of the observed differences in the optimal inhibitory concentrations of the test compounds in non initiated and HER-2/neu oncogene initiated human mammary epithelial cells, appropriate high doses of the test compounds were selected for subsequent experiments.

6. **Effect of I3C on 184-B5/BP, 84-B5/HER and MDA-MB-231 cells: In a recently completed study (Telang et al Proc. Soc. Exp. Biol. Med. 1997, in press, see also appended data.), the antiproliferative activity of I3C was examined on initiated and tumor derived human mammary cells in an effort to identify possible mechanism(s) of action. 184-B5/BP and 184-B5/HER cells represented the initiated cells, while tumor derived MDA-MB-231 represented the transformed cells. Cell cycle progression, E_2 metabolism, cellular apoptosis and anchorage dependent growth constituted the quantitative parameters for perturbation of SEPB.**

In these experiments FACS assay was used for the cell cycle analysis and for determining the extent of Sub G_0 (apoptotic) peak according to the methodology published previously (22,30,31). The relative extent of E_2 metabolism was determined using the radiometric assay (10,13,14,43), and the data was expressed as the ratio of 2-OHE_1/16∝-OHE_1 formation. The extent of modulation in aberrant proliferation was evaluated using the AD-CFE (38). These data were expressed as % colony forming efficiency.

The initiated 184-B5/BP and 184-B5/HER, and transformed MDA-MB-231 phenotypes exhibited a 55-67% decrease in the ratio of quiescent ($Q=G_0$)/proliferative (P=S+M) phases of the cell cycle and a 76-106% increase in ADG-CFE relative to that observed in the non initiated, non tumorigenic 184-B5 cells. The aberrant proliferation was also associated with a 72-90% decrease in cellular apoptosis. At the biochemical level, 184-B5/BP, 184-B5 HER and MDA-

MB-231 cells exhibited a 88-90% decrease in the ratio of 2-OHE_1/16∝-OHE_1 formation relative to that observed in the 184-B5 cells.

To examine the modulatory influence of I3C on the perturbed SEPB, the three cell types were treated for 24hr for the FACS assay, 48 hr for the E_2 metabolism assay and 21 days for the AD-CFE assay. The dose of 50μM I3C was selected because of its substantial growth inhibitory activity as evidenced by the dose response experiments.

Exposure of 184-B5/BP, 184-B5/HER and MDA-MB-231 cells to I3C resulted in a 137%-210% increase in the Q/P ratio, a 4-18 fold increase in 2-OHE_1/16∝-OHE_1 ratio and a 54-61% decrease in the AD-CFE.

These data indicate that I3C may prevent post initiational events and progressional events of carcinogenesis in part due to its ability of regulate aberrant cell cycle progression and enhance the formation of 2-OHE_1, an antiproliferative metabolite of E_2.

7. **Effect of EGCG on 184-B5/HER Cells: The preneoplastically transformed HER-2/neu expressing 184-B5/HER cells were utilized in a recently completed study (Telang et al Breast Cancer Res. Treat. 41:264, Abst#318, 1996, manuscript in preparation, see also appended data) to examine whether EGCG inhibits HER-2/neu induced aberrant proliferation. Cell cycle progression, cellular apoptosis and altered expression of cell cycle regulatory gene products PCNA, cdks (p16), Bcl-2 and p53 represented quantitative parameters to measure the perturbation of SEPB.**

A continuous 7-day exposure of 184-B5/HER cells to 0.22, 2.2, 4.4, 11.0 and 22μM EGCG resulted in 2.8, 30.8, 61.3, 92.0 and 97.9% inhibition of aberrant proliferation, respectively. In subsequent experiments, therefore, a single 24hr. treatment with 22μM EGCG was used. This treatment produced about 5-10% growth inhibition.

The cell cycle analysis revealed a confluency dependent specificity of the effects of EGCG on 184-B5/HER cells. In 25% confluent (log phase) cultures, EGCG treatment induced a 58.7% increase (p=0.001) in cells at G_0 phase with a concomittant 30.4% (p=0.02) and 69.6% decrease (p=0.001) in S and M phase of the cell cycle. In contrast, the 100% confluent (lag phase) cultures responded to EGCG by exhibiting a 36.3% decrease (P=0.001) in the G_0 population, with a concomittant,75.3% (p=0.02) and 140.7% (P=0.001) increase in S and M phases of the cell cycle respectively. The possibility that EGCG may inhibit aberrant cell cycle progression by inducing apoptotic and/or mitotic cell death is being examined at present.

The experiment to examine the effect of EGCG on select cell cycle regulatory gene product expression utilized altered immunoreactivity to tyrosine kinase, PCNA, cdk, Bcl-2 and p53 as quantitative parameters of SEPB. EGCG treatment produced a 56.3% (p=003) and a 65.7% (P=0.009) inhibition in immunoreactivity to HER-2/neu and tyrosine kinase specific antibodies respectivily, indicating that EGCG may inhibit oncogene expression by inhibiting tyrosine kinase activity. The regulation of aberrant proliferation in 184-B5/HER cells by EGCG is evident by a 25-30% inhibition of PCNA and p16 (cdk) immunoreactivity.

Treatment of 184-B5/HER cells with 22μM EGCG resulted in about 3-5 fold increase in cellular apoptosis as evidenced by the presence of Sub G_0 (apoptotic) peak, positive immunoreactivity to apoptosis specific antibody and presence of apoptotic bodies. This induction was accompanied by a 36% inhibition (P=0.002) of Bcl-2 immunoreactivity. These data suggest that EGCG in the present system may induce Bcl-2 dependent apoptosis.

8. **Effect of GEN on 184-B5/HER Cells: The preventive efficacy of GEN was examined in 184-B5/HER cells in a recently completed study (Katdare et al In Vitro Cell. Dev. Biol. 1997, see also appended data). Cell cycle progression, cell cycle regulatory gene product expression and cellular apoptosis represented the quantitative parameters to evaluate the modulation in SEPB. For the initial dose-response studies, GEN at the concentrations of 1.0, 2.5, 5.0, 7.5 and 10μM was used in a 7-day growth assay. The 184-B5/HER cells upon a continuous 7-day exposure exhibited 22.5%, 43.6%, 53.2%, 61.6% and 73% inhibition of aberrant proliferation relative to that observed in 0.1% DMSO treated solvent controls. For the subsequent experiments treatment of 10μM GEN for 24hr. was used. This treatment produced a 8-10% inhibition of growth.**

The cell cycle analysis revealed a confluency dependent specificity of GEN similar to that observed in the experiments with EGCG. In log phase (25% confluent) cultures GEN treatment induced a 425% increase (P=0.001) in G_0/S+M ratio, indicating an increase of quiescent population over that of proliferatively active population of 184-B5/HER cells.

The experiment to evaluate the modulation in expression of cell cycle regulatory gene products demonstrated that GEN treatment resulted in a 77.2% decrease (P=0.001) in tyrosine kinase immunoreactivity, and a 29.2% and a 32.6% decrease (P=0.04) in immunoreactivity to PCNA and cdks respectively.

The experiment designed to examine the effect of GEN on cellular apoptosis, revealed a confluency dependent induction of apoptosis as evidenced by the presence of Sub G_0 peak, immunoreactivity to apoptosis specific apoptag and presence of apoptotic bodies. In log phase (25% confluent) cultures GEN induced a 5.5 fold increase (P=0.001) in Sub G_0 peak, while in lag phase (100% confluent) cultures the induction was about 1.5 fold over the extent of constitutive apoptosis.

The induction of apoptosis was accompanied by a 37.4% decrease (P=0.004) in immunoreactivity to Bcl-2 protein.

The data generated from this study indicates the preventive efficacy of GEN may, in part, be due to its ability to inhibit aberrant cell cycle progression and HER-2/neu associated tyrosine kinase activity and to induce Bcl-2 dependent apoptosis in 184-B5/HER cells.

9. **Modulation of BP-induced Carcinogenesis in184-B5 cells: The ability of the phytochemicals I3C, EGCG and GEN to down-regulate BP-induced carcinogenesis was examined by utilizing the newly developed assays for cell cycle progression, cell cycle regulatory gene product expression and cellular apoptosis (Telang et al Int. J. Oncol. 9:850(Abst#186, 1996, manuscripts in preparation).**

The 184-B5/BP cells (initiated for carcinogenesis by 24hr. treatment with 39μM BP) exhibited impaired homeostasis as evidenced by increased proliferation (low G_0/S+M ratio) and decreased apoptosis (low Sub G_0 peak).

To examine the effects of phytochemicals on BP induced aberrant hyperproliferation, 184-B5/BP cells were treated for 24hr. with highest cytostatic doses of 50μM I3C, 22μM EGCG or 10μM GEN. This treatment protocol produced about 8-10% inhibition of growth. The antiproliferative effect of the three phytochemicals was evident by a 75%, 97% and 97% growth inhibition of 184-B5 cells in a 7-day growth inhibition assay. The treatment of 184-B5/BP cells with the three phytochemicals resulted in variable inhibition of S and/or G_2+M phases of the cell cycle.
The expression of proliferation specific gene products such as PCNA and cdks (p16) was found to be decreased in response to the treatment with the phytochemicals. This alteration in immunoreactivity corresponded with regulation of aberrant cell cycle progression.

The exposure to the three phytochemicals also induced a variable extent of cellular apoptosis as evidenced by increased Sub G_0 fraction. This induction was accompanied by increased immunoreactivity to p53 protein.

Taken together the data generated from the study on 184-B5/BP cells indicates that preventive efficacy of the three phytochemicals on BP induced carcinogenesis may, in part, be due to their ability to regulate aberrant cell cycle progression and induce p53-dependent apoptosis.

The data generated from the experiments utilizing the human mammary epithelial cell culture system have demonstrated that the three phytochemicals affect the aberrant proliferation detectable in initiated cells. The preventive efficacy is predominantly in the post-initiaional or promotional phase of the tumorigenic transformation. The mechanisms involved in prevention by the phytochemicals

may differ depending upon the initiator of transformation (chemical carcinogens or oncogene) and may be distinct for individual preventive agent. This aspect needs to be systematically pursued.

V. GENERAL CONCLUSIONS:

The recently completed experiments discussed in this report together with the ongoing projects are designed to identify molecular and cellular targets for human mammary carcinogenesis, and to elucidate the possible mechanisms of action for preventive efficacy of naturally-occurring tumor inhibitors. This approach provides evidence for utility of the present *in vitro* models and surrogate endpoint biomarkers as assay systems to identify potential human carcinogens and evaluate the preventive efficacy of new naturally-occurring or synthetic compounds.

During the two year funding of this research program, every effort was made to accomplish all of the tasks detailed in the original Statement of Work. The proposed experiments with β-C, EGCG and GEN on the TDLU explant culture model were discontinued after the dose response studies, in part because of limited solubility of the compounds in conventional solvent systems, and inadequate availability of surgical samples from pre-menopausal patients at specific phase of the menstrual cycle. The projects on the explant culture model were replaced by those utilizing the 184-B5 cell culture model.

During the second year funding, experiments were designed to enhance the specificity of biomarker assay for E_2 metabolism. These experiments included optimization of reverse phase high pressure liquid chromatography (RP-HPLC) and gas chromatography-mas spectrometry (GC-MS) methods for separation and quantitation of estradiol metabolites from the 184-B5, 184-B5/BP and 184-B5/HER cell culture medium. In addition, the biomarker assays for aberrant hyperproliferation were expanded to include cell cycle analysis, immunoreactivity to cell cycle regulatory gene products and cellular apoptosis. These experiments involved optimization of fluorescence-assisted cell sorting (FACS) for flow cytometry, and quantitative fluorescence microscopy for cellular epifluorescence. The ongoing experiments have utilized the newly developed SEPB assays to examine the preventive efficacy of EPA, I3C, EGCG and GEN on 184-B5/BP, and 184-B5/HER cells. The data generated from these experiments have been analyzed and presented at several research conferences. In addition, full length manuscripts are either published, accepted for publication or are being prepared for submission to peer reviewed journals.

It is conceivable that sequence of events leading to modulation of growth of initiated (preneoplastic) and pre-invasive phenotype may be a culmination of multiple molecular and biochemical events. A systematic analysis of cell cycle regulation, cellular apoptosis and cytodifferentiation, in response to known initiators of carcinogenesis, and modulatory influences of naturally-occurring or synthetic agents on the carcinogenic process should provide important leads for clinically relevant preventive interventions. Promising agents identified using this approach could then be screened for lack of toxicity, enhanced

efficacy and superior specificity by conventional pre-clinical or phase I, II and III clinical trials.

VI. PUBLICATIONS (1994-1997)

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3. Fishman J, Osborne MP, Telang NT. The role of estrogen in mammary carcinogenesis. Ann NY Acad Sci 768:91-100; 1995

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13. Telang NT, Katdare M, Braldow HL, Osborne MP, Fishman J. Inhibiiton of proliferation and modulation of estradiol metabolism: Novel mechanisms for breast cancer prevention by the phytochemical indole-3-carbinol. Proc. Soc. Exp. Biol. Med. 1997 (in press).

VII. PERSONNEL:

The following investigators have collaborated with the Principal Investigator during the two year funding of this research program.

NAME	TIME & EFFORT	SALARY
Nitin T. Telang (Principal Investigator)	20	Yes
Michael P. Osborne (Co-Investigator)	5	No
George Y. C. Wong (Co-Investigator)	5	No
H. Leon Bradlow (Co-Investigator)	5	No
Meena Katdare (Research Fellow)	50	No
Milan Zvanovec (Research Technician)	25	Yes

Except for the P.I. and the Research Technician, none of the collaborators have received salary support from this negotiated effort.

VIII. MANUSCRIPTS IN PREPARATION

1. Katdare M, Steiner M, Osborne MP, Telang NT. Altered cell cycle regulation and modulated cellular apoptosis in human mammary epithelial cells: surrogate endpoint biomarkers for prevention of carcinogenesis. (Cytometry)

2. Katdare M, Staino-Coico L, Osborne MP, Telang NT. Induction of apoptosis by genistein in HER-2/neu oncogene transformed human mammary epithelial cells: a mechanism for chemoprevention. (Cancer Res)

3. Telang NT, Katdare M, Osborne MP. Aberrant hyperproliferation and cellular apoptosis: Modifiable surrogate endpoint biomarkers for cancer chemoprevention. (Int J Oncol)

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5. Katdare M, Dannenberg AJ, Osborne MP, Telang NT. Curcumin inhibits c-myc oncogene-induced tumorigenic transformation in mammary epithelial cells. (Cancer Lett).

6. Jinno H, Katdare M, Osborne MP, Telang NT. Negative growth regulation of oncogene-transformed human mammary epithelial cells: Role of apoptosis. (Breast Cancer Res. Treat.)

IX. APPENDIX MATERIAL

Grant Number DAMD 17-94-J-4208

"Prevention of Human Mammary Carcinogenesis"

1. **Legends For Appendices:**

Appendix Table 1 (AT-1): Biomarker assays for prevention of mammary carcinogenesis. The table presents assays utilized and endpoints measured. These represent quantitative parameters specific for evaluation of prevention efficacy of phytochemicals.

Appendix Figure (AF-1): A. Status of Ras-p21-GTP binding in the BP-initiated TDLU explant culture system. The extent of p21 protein bound to [α^{32}P] determined from 4 independent patient samples is shown. B. Status of 16α-OHE$_1$ formation in BP-initiated TDLU explant culture system from 4 independent patient samples is shown.

Apendix Figure 2 (AF-2): A. Modulation of replicative DNA synthesis as a marker for aberrant proliferation is evaluated by determining incorporation of ^{3}H-thymidine into cellular DNA. The aberrant proliferation induced by BP is down-regulated in the presence of either EPA or I3C. B. Modulation of E_2 metabolism as determined by the ratio of C2/C16α-hydroxylation of E_2. Treatment with BP resulted in decreased ratio, while a simultaneous treatment with BP +EPA or BP +I3C abrogated the effect of BP.

Appendix Table 2 (AT-2): The genotoxicity of DMBA and BP is evaluated in 184-B5 cells be determining the formation of purine nucleotide adducts.

Appendix Table 3 (AT-3): The growth characteristics of 184-B5 cells initiated by the HER-2/neu oncogene are compared with the parental 184-B5 cells by determining population doubling times, saturation density and cell cycle distribution.

Appendix Table 4 (AT-4): The 184-B5 cells upon initiation with BP or with HER-2/neu exhibit enhanced proliferation relative to that observed in non initiated cells. This alteration is evidencedx by increased number of cells in proliferative (S and/or M) phases of the cell cycle.

Appendix Table 5 (AT-5) Constitutive expression of selected proliferation specific, apoptosis specific and differentiation specific gene products is compared in the parental 184-B5 cells and HER-2/neu initiated 184-B5/HER cells.

Appendix Table 6 (AT-6): The status of cellular apoptosis is compared in 184-B5 (parental, non initiated), 184-B5/BP (BP initiated) and 184-B5/HER (HER-2neu initiated) cells. In the initiated cells apoptosis is decreased relative to that seen in non initiated cells. Inset: cell cycle profile of 184-B5 cells showing distinct peaks from cells in Sub G_0, G_0/G_1, S and G_2/M phases of the cell cycle.

Appendix Figure 3A(AF-3A): The stages of apoptosis detectable in 184-B5 cells are presented. These include nuclear condensation, membrane blebbing, nuclear fragmentation and apoptotic bodies. (H&E staining, 100x magnification).

Appendix Figure 3B (AF-3B): The status of apoptosis in 184-B5 cells as demonstrated by cultures stained with Apoptag (A,C) and with propidium iodine (B,D). By both the procedures nuclear fragmentation is clearly detectable.(Dark field fluorescence, 100x magnification).

Appendix Figure 4 (AF-4): Effect of I3C on 184-B5/BP, 184-B5/HER and MDA-MB-231 cells is presented. The effects are quantified by dtermining Q/P ratio, E_2 metabolism, cellular apoptosis and anchorage-dependent colony forming efficiency.

Appendix Figure 5 (AF-5): Effect of (-) epigallo catechin gallate (EGCG) on 184-B5/HER cells is presented. The inhibitory effect of EGCG on aberrant proliferation of 184-B5/HER cells is quantified by dose response determination, cell cycle analysis, cellular apoptosis and status of immunoreactivity to select cell cycle regulatory gene products.

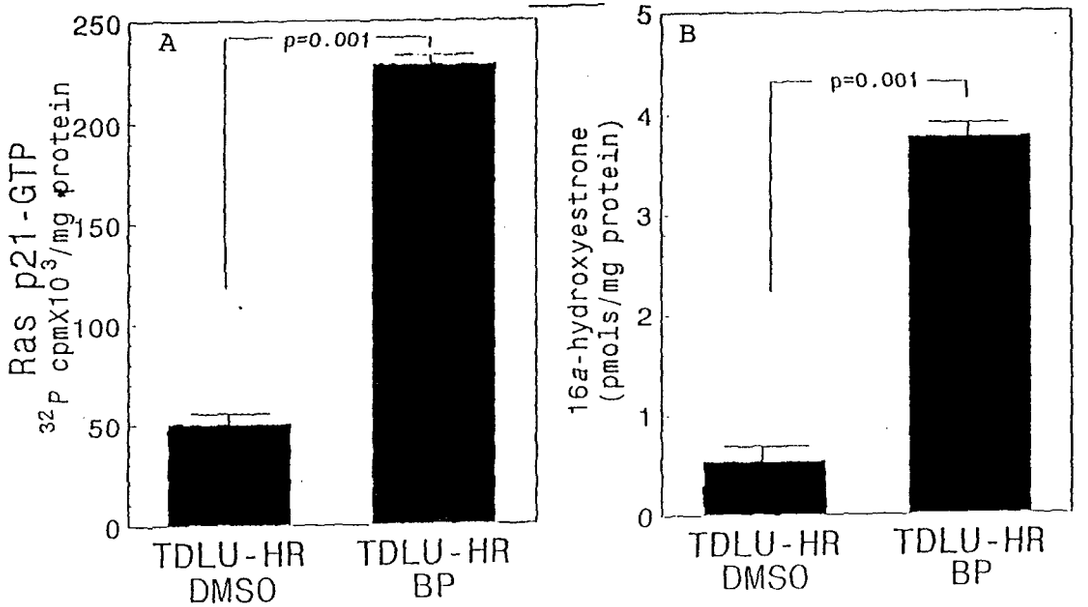
Appendix Figure 6 (AF-6): Effect of Genistein (GEN) on 184-B5/HER cells is presented. The experiments are designed to examine dose dependent inhibition of aberrant proliferation, influence of cytostatic dose of GEN on cell cycle progression, induction of apoptosis and altered immunoreactivity to the antiapoptotic Bcl-2 gene product.

BIOMARKER ASSAYS FOR PRIMARY PREVENTION

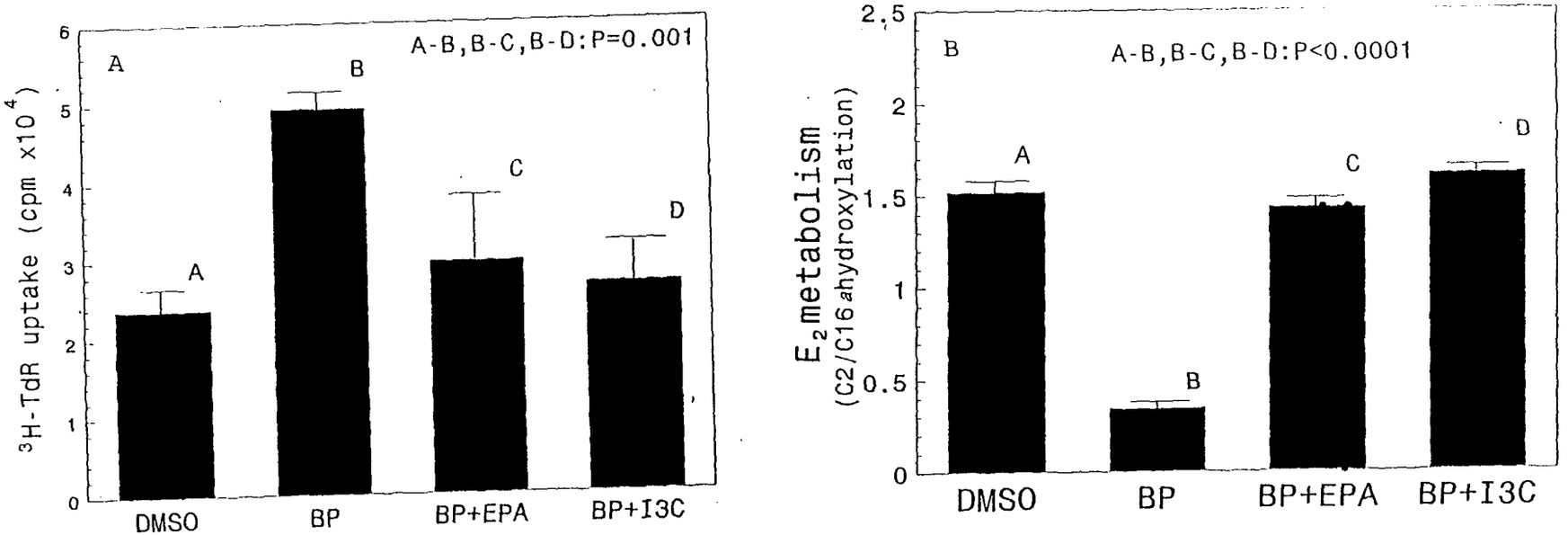
Biomarker	Assay	Endpoint
Biochemical	[^{33}P] post labeling	purine nucleotide adducts
	HU-insensitive [^{3}H] thymidine uptake	unscheduled DNA synthesis
	GC-MS for E_2 metabolites	E_1, 16α-OHE_1, 2-OHE_1 levels
Cellular	FACS analysis for cell cycle progression	PI, AO positivity
	Apoptosis	Apoptag positivity
Cytodifferentiation	immunocytochemistry, cellular epifluorescence	cyp1A1, GST, β-casein, cytokeratin, ER, PR positivity

GC-MS: Gas Chromatography-Mass Spectrometry; FACS: Fluorescence-assisted Cell Sorting; cyp1A1: Cytochrome P450 1A1; GST: Glutathione-S-transferase; PI: Propidium Iodide; AO: Acridine Orange; ER: Estrogen Receptor; PR: Progesterone Receptor

AF-1


A
p=0.001
Ras p21-GTP
^{32}P cpmX10^3/mg protein
250
200
150
100
50
0
TDLU-HR DMSO
TDLU-HR BP
B
p=0.001
16a-hydroxyestrone (pmols/mg protein)
5
4
3
2
1
0
TDLU-HR DMSO
TDLU-HR BP

AF-2


A-B,B-C,B-D:P=0.001
^{3}H-TdR uptake (cpm X10^4)
A
B
C
D
6
5
4
3
2
1
0
DMSO
BP
BP+EPA
BP+I3C
B
A-B,B-C,B-D:P<0.0001
E_2 metabolism (C2/C16ahydroxylation)
2.5
2
1.5
1
0.5
0
A
B
C
D
DMSO
BP
BP+EPA
BP+I3C

AT - 2

CARCINOGEN-DNA ADDUCT FORMATION IN HUMAN MAMMARY EPITHELIAL 184-B5 CELLS

Treatment[a]	DNA Content ($\mu g/10^6$ Cells)	Purine Nucleotide Adducts[b] (p mole/mg DNA)
None	6.9	ND
DMSO (solvent)	7.0	ND
DMBA	8.4	0.39 ± 0.13[c]
BP	9.1	1.74 ± 0.44[c]

[a] cell cultures treated with 0.1% DMSO, 39 μM DMBA or 39 μM BP for 24 hours.

[b] HPLC analysis of [^{33}P] post labeled cellular DNA. Adducts identified as diole expoxides of d-Gou and d-Ado formed from DMBA and BP. ND: Not Detectable

[c] mean ± SD, n = 9

AT - 3

GROWTH CHARACTERISTICS OF HUMAN MAMMARY EPITHELIAL 184-B5 CELLS

Initiator	Population Doubling Time[a] (Hours)	Saturation Density[b] ($\times 10^5$)	% Distribution of Cells[c]		
			$G_0 + G_1$	S	$G_2 + M$
None	32.8 ± 1.6	23.8 ± 2.9	63.2 ± 2.3	14.2 ± 3.1	4.5 ± 0.3
HER-2/neu	21.6 ± 2.1	37.6 ± 7.2	54.9 ± 7.4	32.2 ± 3.6	5.6 ± 0.3

[a] determined from the linear portion of the growth curves in log phase cultures

[b] cell number obtained from ≈ 90% confluent cultures at day 9 after seeding 1.0×10^5 cells per 25 cm^2.

[c] Analysis of propidium iodide stained cell suspension by fluorescence-assisted cell sorting (FACS-SCAN).

AT-4

Cell Cycle Analysis of Human Mammary Epithelial Cells

Cell Line	% Distribution of Cells [a, b]		
	$G_0 + G_1$	S	$G_2 + M$
184-B5	64.0 ± 5.1	17.5 ± 4.0 [c]	7.5 ± 4.2 [f]
184-B5/BP	61.5 ± 9.4	32.3 ± 2.4 [d]	17.6 ± 1.6 [g]
184-B5/HER	54.9 ± 7.4	32.2 ± 3.6 [e]	5.6 ± 0.3 [h]

[a] Cell suspensions from semi-confluent cultures were stained with propidium iodide and analyzed by the fluorescence-assisted cell sorting (FACS) assay. Approximately 1.0×10^4 cells were monitored for the phases of cell cycle.

[b] mean ± SD, n = 8

[c-d, c-e] P = 0.001

[f-g] P = 0.01

[f-h] n.s.

AT-5

Status of Cell Cycle Regulatory Genes in Human Mammary Epithelial Cells

Cell Cycle Regulatory Gene Product	Immunofluorescence (Mean fluorescence Intensity) [a]	
	184-B5	184-B5/HER
HER-2/neu	ND	9.8 ± 0.5
Py20	ND	38.3 ± 4.9
Proliferation Specific		
PCNA	68.8 ± 2.3	8.8 ± 0.1
Cyclin D1	6.4 ± 0.1	10.3 ± 0.5
p16 (cdk 4, 6)	8.7 ± 0.2	10.7 ± 1.0
cdk 7	2.6 ± 0.1	11.2 ± 0.1
Apoptosis Specific		
Bcl-2	89.1 ± 10.7	69.2 ± 3.6
p53	2.2 ± 0.1	15.3 ± 5.3
Apo-1/fas	3.3 ± 0.1	9.4 ± 3.8
Differentiation Specific		
EGFR	19.3 ± 0.6	65.3 ± 14.3
CAM 5.2-ck	4.6 ± 0.2	8.3 ± 0.3

[a] mean ± SD, n = 4

AT-6

Status of Cellular Apoptosis in Human Mammary Epithelial Cells

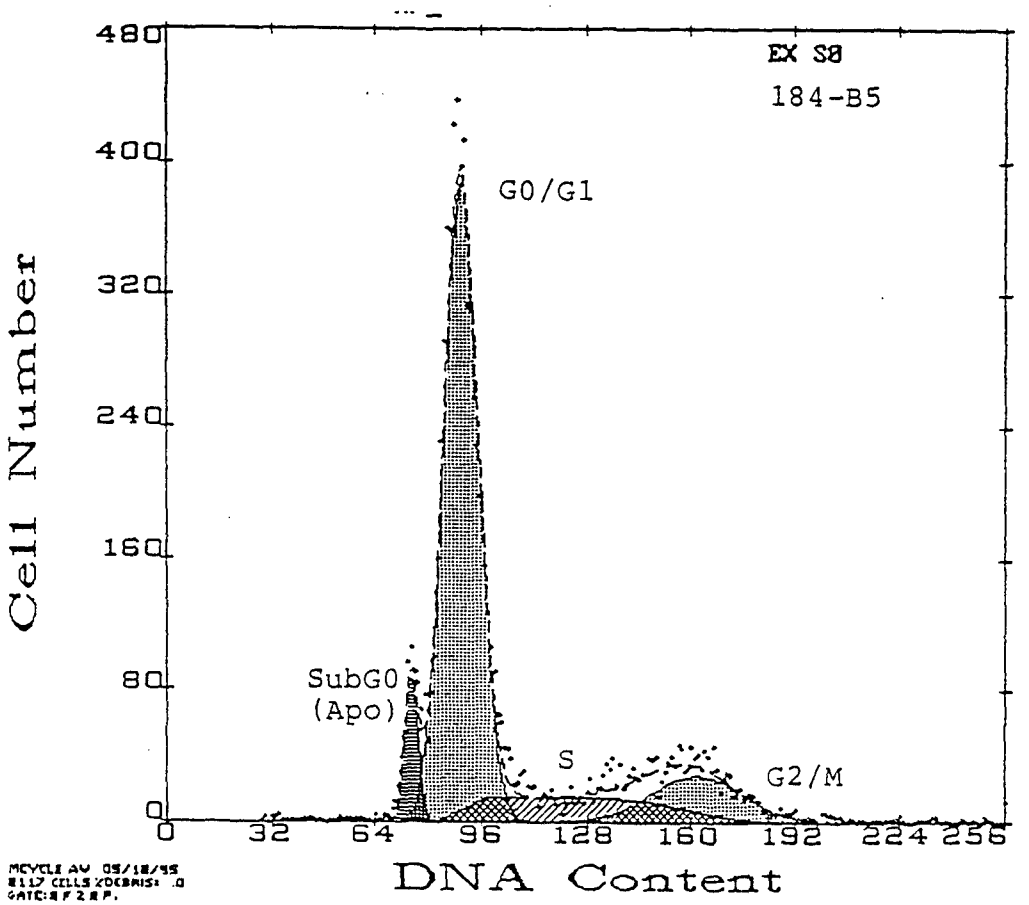
Cell Line	% Apoptotic Cells [a]	Relative Decrease (% of Control)
184-B5	16.2 ± 2.7 [b]	-
184-B5/BP	1.9 ± 0.9 [c]	88.3
184-B5/HER	5.7 ± 1.6 [d]	64.8

[a] determined from the intensity of sub G_0 (apoptotic) peak after FACS analysis of propidium iodide stained cell suspension.

[b-d] mean ± SD, n = 8

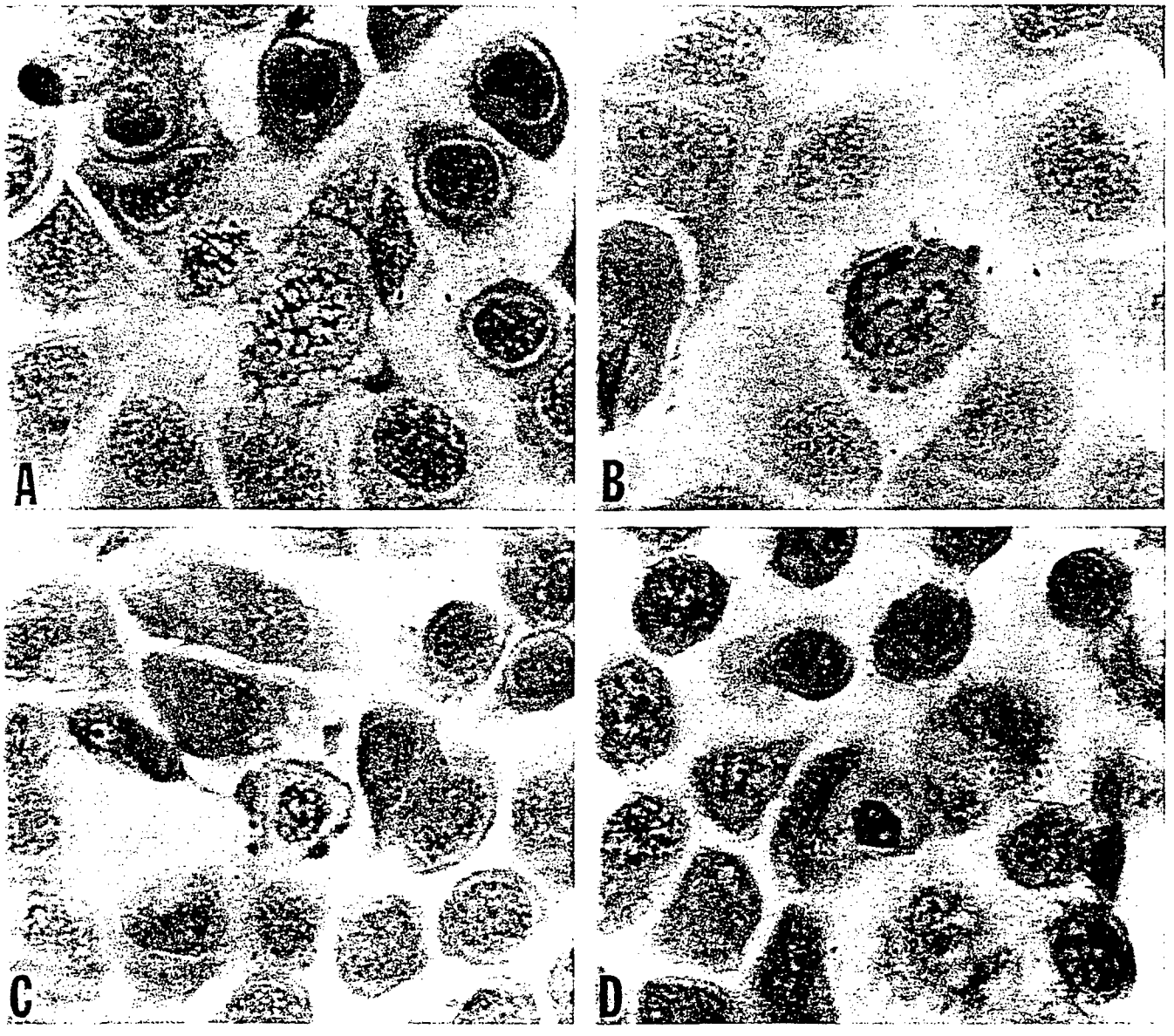
[b-c] P = 0.0001

[b-d] P = 0.001



AF-3A

CELLULAR APOPTOSIS IN 184-B5 CELLS



A: Nuclear Condensation

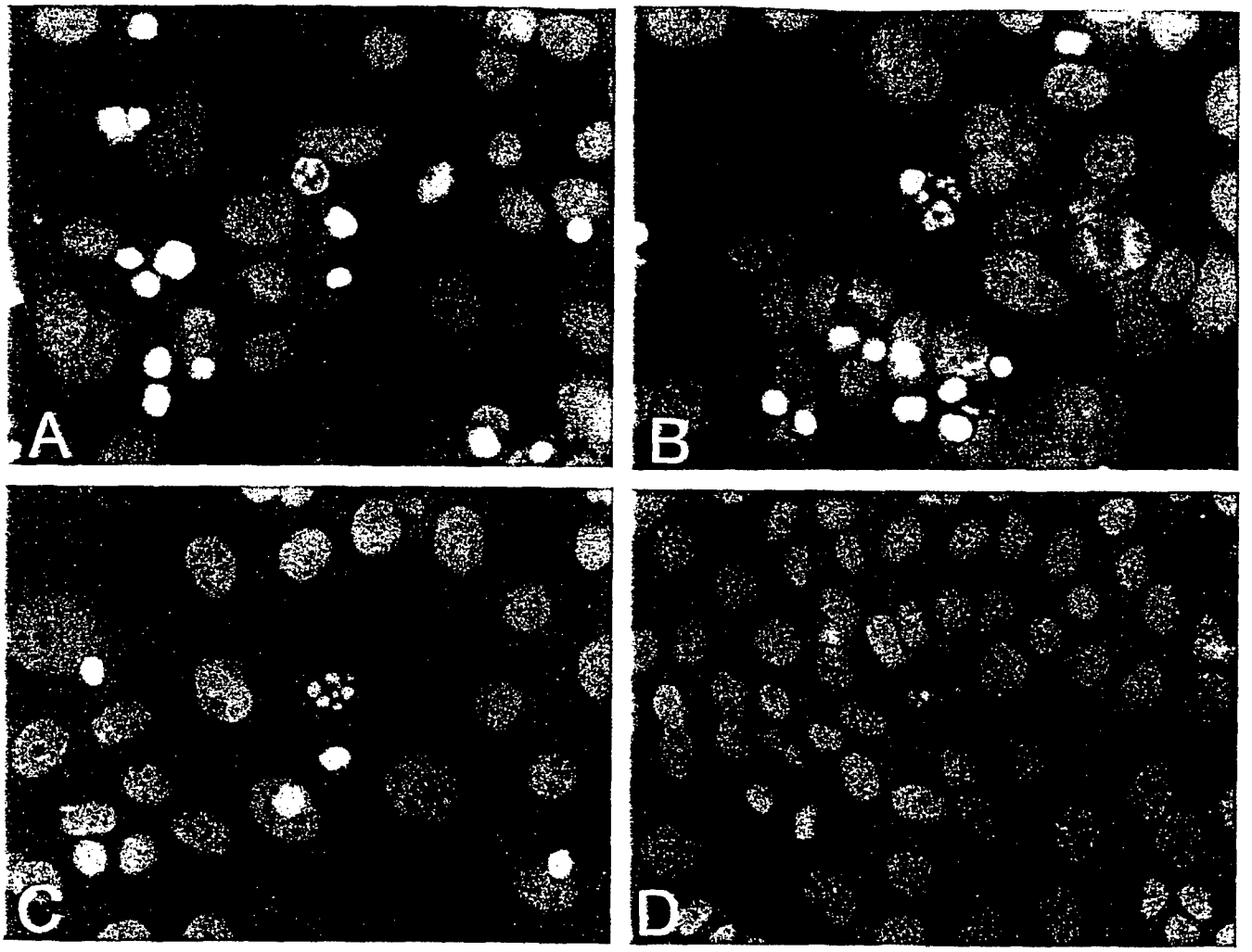
B: Membrane Blebbing

C: Nuclear Fragmentation

C: Apoptotic Body

AF-3B

CELLULAR APOPTOSIS IN 184-B5 CELLS: EPIFLUORESCENCE



A: Apoptag Positivity

B: Propidium Iodide Positivity

C: Nuclear Fragmentation

D: Nuclear Fragmentation

Effect of Indole-3-Carbinol on Cell Cycle Regulation in Human Mammary Epithelial Cells

Cell Line	Treatment	% Distribution of Cells [a,b]		
		Quiescent Phase (G_0)	Proliferative Phase (S+M)	Q/P Ratio[c]
184-B5/BP	DMSO	43.8±2.1	56.2±2.1	0.78±0.70
	I3C	70.2±2.9	29.8±2.9	2.38±0.33
184-B5/HER	DMSO	45.6±23.3	54.4±3.3	0.84±0.11
	I3C	65.8±3.3	34.2±6.3	1.99±0.56
MDA-MB-231	DMSO	36.1±1.6	63.9±1.6	0.59±0.05
	I3C	64.6±2.1	35.4±2.1	1.83±0.20

[a] determined from flow cytometry of propidium iodide-stained cell suspension.

[b] Values are mean ± SD, n=4 per treatment group.

[c] 184-B5/BP: p=0.001; 184-B5/HER: p=0.004; MBA-MD-231: p=0.001.

Modulation of Estradiol Metabolism by Indole-3-Carbinol in Human Mammary Epithelial Cells

Cell Line	Treatment	Estradiol metabolism [a,b] (p/mole/10^4cells/48hr)		
		2-OHE_1	16∝-OHE_1	2/16∝ Ratio [c]
184-B5/BP	DMSO	1.3±0.2	2.2±0.2	0.6±0.2
	I3C	12.7±0.2	1.4±0.2	9.2±1.4
184-B5/HER	DMSO	1.4±0.2	2.7±0.1	0.5±0.2
	I3C	11.5±0.7	1.2±0.3	9.7±2.1
MDA-MB-231	DMSO	1.5±0.2	3.8±0.7	0.4±0.1
	I3C	3.6±0.6	1.9±0.2	2.1±0.3

[a] determined by the radiometric assay measuring $^3H_20$ formation after a 48hr. incubation with [C2-^{3}H] E_2 or [C16∝-^{3}H] E_2 in the presence of 0.1% DMSO or 50 μM I3C.

[b] metabolite concentrations calculated from stoichiometric convertibility of specifically labeled E_2. Values are mean ± SD, n=18 per treatment group.

[c] 184-B5/BP: p=0.001; 184-B5/HER: p=0.004; MBA-MD-231: p=0.001.

Induction of Apoptosis in Human Mammary Epithelial Cells by Indole-3-Carbinol

Cell Line	Treatment	% Apoptosis [a,b]	p
	DMSO	1.8±0.5	--
184-B5/BP	I3C	5.6±0.6	0.04
	DMSO	5.3±0.6	--
184-B5/HER	I3C	16.1±2.5	0.01
	DMSO	2.9±0.4	--
MDA-MB-231	I3C	6.9±0.5	0.01

[a] determined from the extent of $SubG_0$ (apoptotic) phase using flow cytometry of propidium iodide-stained cell suspension.

[b] Values are mean ±SD, n=4 per treatment group.

Inhibition of Anchorage-dependent Growth of Human Mammary Epithelial Cells by Indole-3-Carbinol

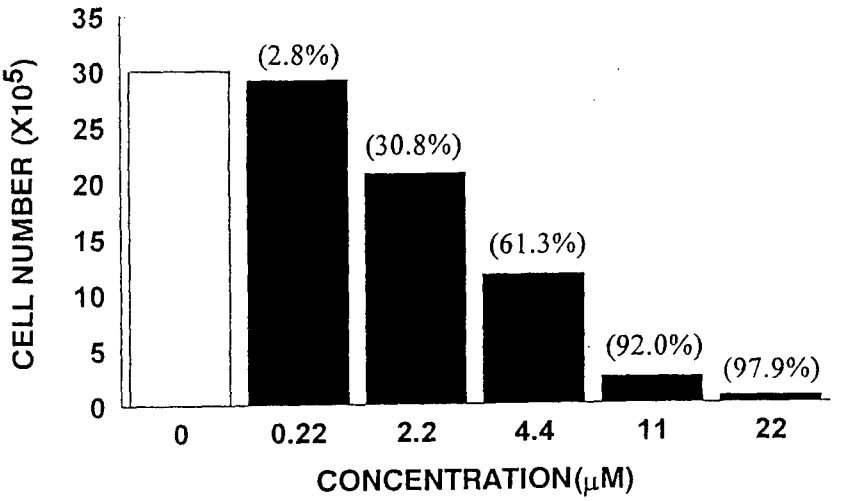
Cell Line	Treatment [a]	Anchorage-dependent Colony Forming Efficiency (AD-CFE)[b,c]
	DMSO	19.3±1.6
184-B5/BP	I3C	8.9±2.1
	DMSO	20.5±1.4
184-B5/HER	I3C	9.2±3.3
	DMSO	23.1±2.2
MDA-MB-231	I3C	9.0±1.2

[a] Cells exposed to 0.1% DMSO or 50 μM I3C for 21 days, and number of anchorage-dependent (adherent) colonies determined.

[b] AD-CFE: $\frac{\text{Number of Colonies}}{\text{Initial Seeding Density}} \times 100$

[c] Values are mean ±SD, n = 18 per treatment group. DMSO vs. I3C. p = 0.01.

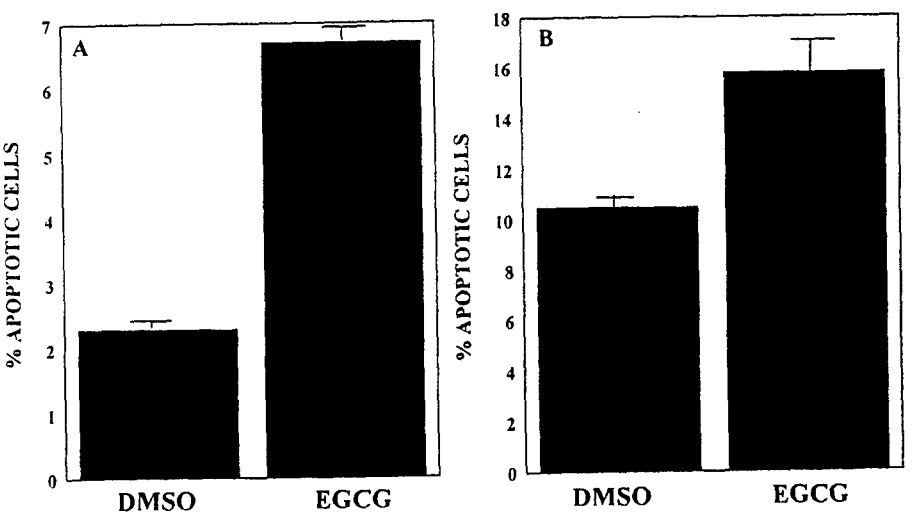
Inhibition of Growth by EGCG in 184-B5/HER Mammary Epithelial Cells

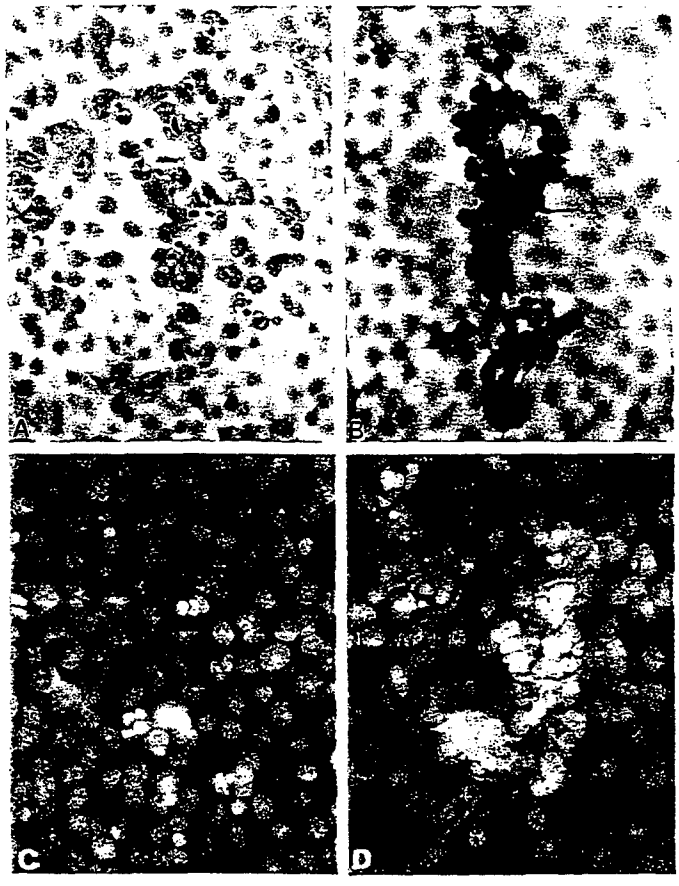


Confluency-dependent Alteration in Cell Cycle Progression of 184-B5/HER Cells by (-)Epigallocatechin Gallate(EGCG)

status of confluency	treatment[a]	% distribution of cells[b, c]		
		G_0+G_1	S	G_2+M
25%	none	45.1±3.9	29.9±3.2	25.0±5.7
	22μM EGCG	71.6±9.6	20.8±1.4	7.6±1.2
100%	none	73.8±8.8	15.4±4.5	10.8±4.6
	22μM EGCG	47.0±6.6	27.0±5.3	26.0±1.4

[a] Cells were treated for 24hours with EGCG dissolved in the culture medium.
[b] Determined from the flow cytometric analysis of propidium iodide-stained cell suspension.
[c] Values are mean±SD, n=8 per treatment group.
G_0+G_1: p=0.001, S:p=0.02, G_2+M: p=0.001



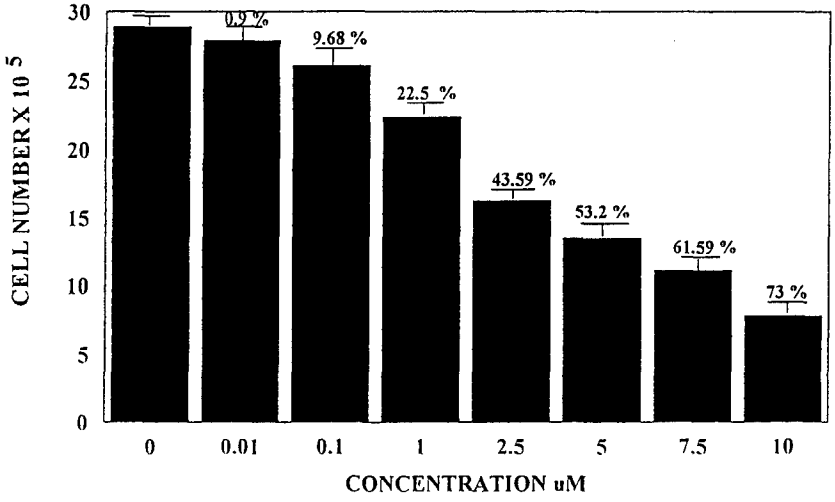


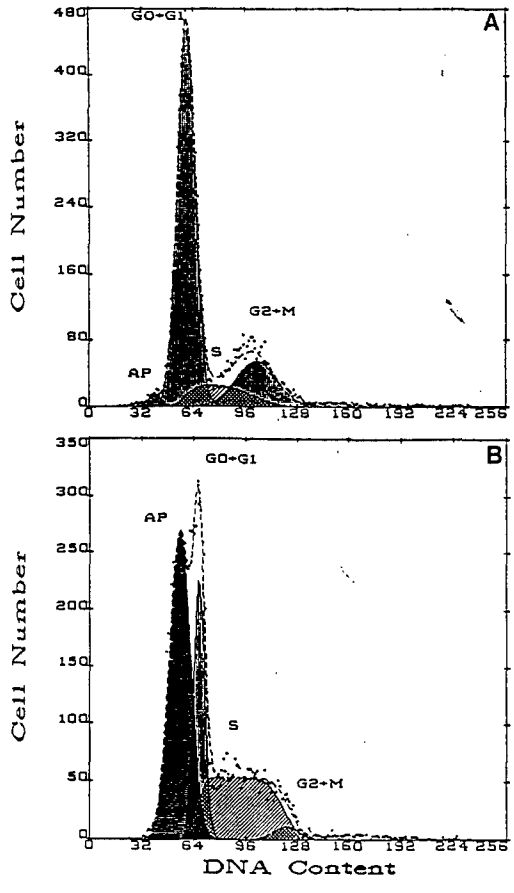
Alteration in immunoreactivity to Cell Cycle Regulatory Gene Products in 184-B5/HER Cells Treated with (-)Epigallocatechin Gallate(EGCG)

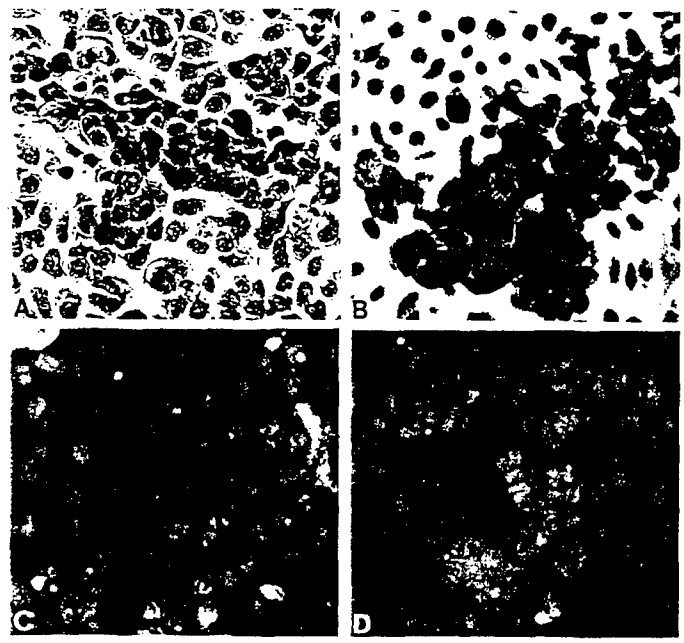
gene product	fluorescence intensity[a, b] (arbitary fluorescence units, AFU)		
	none	EGCG	p
HER-2/neu	22.2±0.7	9.7±0.5	0.003
Py-20	38.2±4.9	13.1±0.3	0.009
PCNA	11.3±0.5	8.5±0.3	0.01
p16	14.1±0.6	9.3±0.3	0.01
Bcl-2	69.2±3.6	43.9±1.4	0.002
p53	15.3±5.2	12.3±4.0	0.20

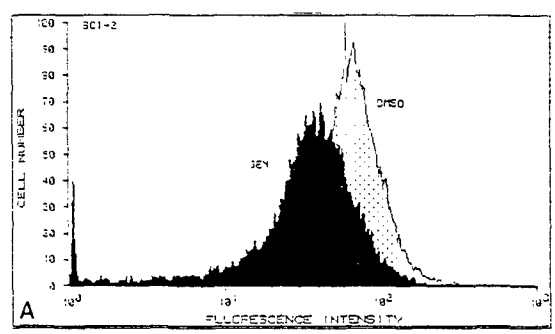
[a] Determined from flow cytometry of cell suspension stained with fluorescein-isothiocyanate(FITC) labeled antibodies.
[b] Values are mean±SD, n=6 per treatment group. Corrected for non-specific fluorescence of FITC labeled IgG.

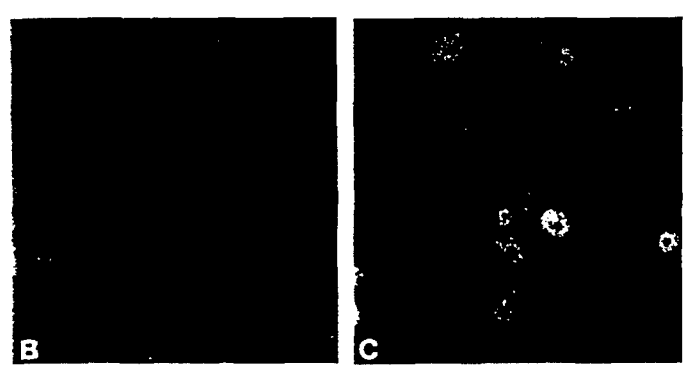
INHIBITION OF GROWTH BY GENISTEIN IN 184-B5/HER HUMAN MAMMARY EPITHELIAL CELLS






A
B
C
D


BCI-2
DMSO
CELL NUMBER
FLUORESCENCE INTENSITY
A


B
C

2. List of Appended Publications:

Appendix Publication 1 (AP-1):

Telang NT, Bradlow HL, Osborne MP. Biomarkers of mammary preneoplastic transformation: Endpoints for cancer chemoprevention. Proc. XVI International Cancer Congress pp. 1285-1290, 1994.

Appendix Publication 2 (AP-2):

Telang NT. In vitro modulation of human mammary carcinogenesis: A model for chemoprevention. Proc. Amer. Assoc. Cancer Res.36:589(Abst.# 3509), 1995.

Appendix Publication 3 (AP-3):

Katdare M, Osborne MP, Talang NT. Chemoprevention of human mammary carcinogenesis by naturally-ocurring phytochemicals. Breast Cancer Res. Treat. 37:28(Abst.#354), 1995.

Appendix Publication 4 (AP-4):

Araki R, Inoue S, Osborne MP, Telang NT. Chemoprevention of mammary preneoplasia: In vitro effects of a green tea polyphenol. Ann. NY Acad. Sci. 768: 215-222, 1995.

Appendix Pubilcation 5 (AP-5):

Fishman J, Osborne MP, Telang NT. The role of estrogen in mammary carcinogenesis. Ann. NY. Acad. Sci 768: 91-100, 1995.

Appendix Publication 6 (AP-6):

Telang NT. Oncogenes, estradiol biotransformation and mammary carcinogenesis. Ann. NY. Acad. Sci. 784:277-287, 1996.

Appendix Publication 7 (AP-7):

Subbaramaiah K, Telang NT, Ramonetti JT, Araki R, DeVito B, Weksler BB, Dannenberg AJ. Transcription of cyclooxygenase-2 is enhanced in transformed mammary epithelial cells. Cancer Res. 56: 4424-4429, 1996.

Appendix Publication 8 (AP-8):

Telang NT, Katdare M, Osborne MP. Negative growth regulation of HER-2/neu oncogene transformed human mammary epithelial cells by a green tea polyphenol. Breast Cancer Res. Treat. 41:204(Abst.#318), 1996.

Appendix Publication 9 (AP-9):

Katdare M, Osborne MP, Telang NT. Role of cell cycle regulation and apoptosis in prevention of human breast cancer by phytochemicals. Proc. symposium on apoptosis and Multidrug Resistance. 24-25, 1997.

Appendix Publication 10 (AP-10):

Telang NT, Katdare M, Bradlow HL, Osborne MP. Estradiol metabolism: An endocrine biomarker for modulation of human mammary carcinogenesis. Env. Health Persp. 1997 (in press)

Appendix Publication 11 (AP-11):

Telang NT, Katdare M, Bradlow HL, Osborne MP, Fishman J. Inhibition of proliferation and modulation of estadiol metabolism: Novel mechanisms for breast cancer prevention by the phytochemical Indole-3-Carbinol. Proc. Soc. Exp. Biol. Med. 1997 (in press)

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XVI INTERNATIONAL CANCER CONGRESS

New Delhi (India), October 30 - November 5, 1994

Editors
R.S. RAO, M.G. DEO and L.D. SANGHVI
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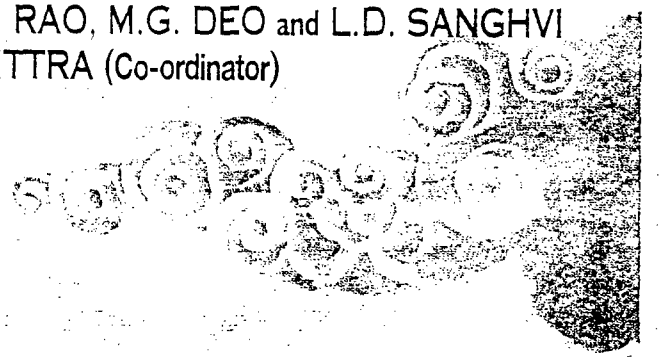

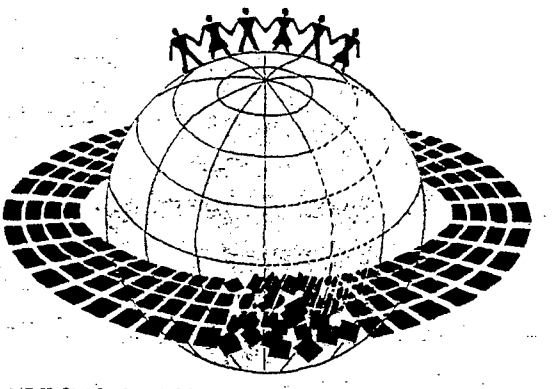

MONDUZZI EDITORE

INTERNATIONAL PROCEEDINGS DIVISION

XVI International
Cancer Congress
1994

New Delhi, India
30 Oct. - 5 Nov. 1994

Biomarkers of mammary preneoplastic transformation: end points for cancer chemoprevention

N.T. TELANG, H.L. BRADLOW
and M.P. OSBORNE

Strang-Cornell Cancer Research Laboratory
Cornell University Medical College, New York (USA)

SUMMARY

In the multistage development of breast cancer initiation and promotion of preneoplasia is an important, early-occurring event representing a marker for risk and for efficacious preventive intervention. In vitro models from non-involved murine and human mammary tissue were utilized to measure i.) upregulation of molecular and endocrine markers in response to treatment with prototype chemical carcinogens and ii.) efficacy of selected naturally-occurring tumor inhibitors in suppressing the perturbed markers. Carcinogen-initiated mammary explant cultures exhibited increased Ras-mediated signal transduction, unscheduled DNA synthesis (UDS) and estradiol 16α-hydroxylation. Treatment of initiated cultures with tumor promoting omega-6 fatty acid **increased**, while that with tumor inhibiting omega-3-datty acid **decreased** the perturbed biomarkers. Thus, induction and modulation of biomarkers prior to tumorigenesis validate their clinical relevance as end points for cancer risk and for efficacious preventive intervention.

XVI International
Cancer Congress
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INTRODUCTION

The conventional predictors of risk for human mammary cancer include demographic, genetic, endocrine and pathologic factors as quantitative end points (1-4). These risk factors are expressed only in 25% of the population that develops breast cancer (2,3). The lack of concordance between the frequency of expression of risk factors and incidence of breast cancer emphasizes a need for identification of better biomarkers.

An in vitro model developed from non-involved murine and human mammary tissue was utilized in the present study to compare the target-tissue susceptibility to prototypic rodent mammary carcinogens, and to evaluate the ability of selected naturally-occurring tumor inhibitors for modulation of carcinogenesis.

MATERIALS AND METHODS

Explant cultures: Tissue fragments with mammary duct epithelium (MDE) from BALB/c mice and with terminal duct lobular units (TDLU) from human mastectomy specimens were used as explant cultures (5-7). The cultures were treated in vitro with metabolism-dependant carcinogens 7,12-dimethylbenz(a) anthracene (DMBA), benzo(a) pyrene (BP) or direct acting carcinogen N-nitroso-N-methylurea (NMU), and were maintained in an humidified atmosphere of 5% CO2: 95% air as described (5,8). The modulators of carcinogenesis that were used as test compounds included the polyunsaturated fatty acids linoleic acid (LNA) and eicosapentaenoic acid (EPA). These naturally-occurring dietary agents modulate mammary tumorigenesis in rodent models (3,8-10).

Biomarkers: Ras p21-GTP binding, C16α-hydroxylation of estradiol and ^{3}H-thymidine uptake were utilized as end points for the biochemical and proliferative markers (5-7).

RESULTS AND DISCUSSION

Perturbation of biochemical markers by chemical carcinogens: To quantify the acute effects of prototype chemical carcinogens, relative extent of UDS, Ras p21-GTP binding and estradiol metabolism was measured in explant cultures of MDE and of TDLU that were treated with DMBA, BP and NMU (Fig. 1a-c). All the three prototype chemical carcinogens effectively increased the extent of three biochemical markers. Consistent with our previous observations (3,5,8), this data indicates that the non-involved mammary tissue is susceptible to DNA damage by rodent mammary carcinogens. Furthermore, initiated target tissue exhibits altered responsiveness to estradiol as evidenced by

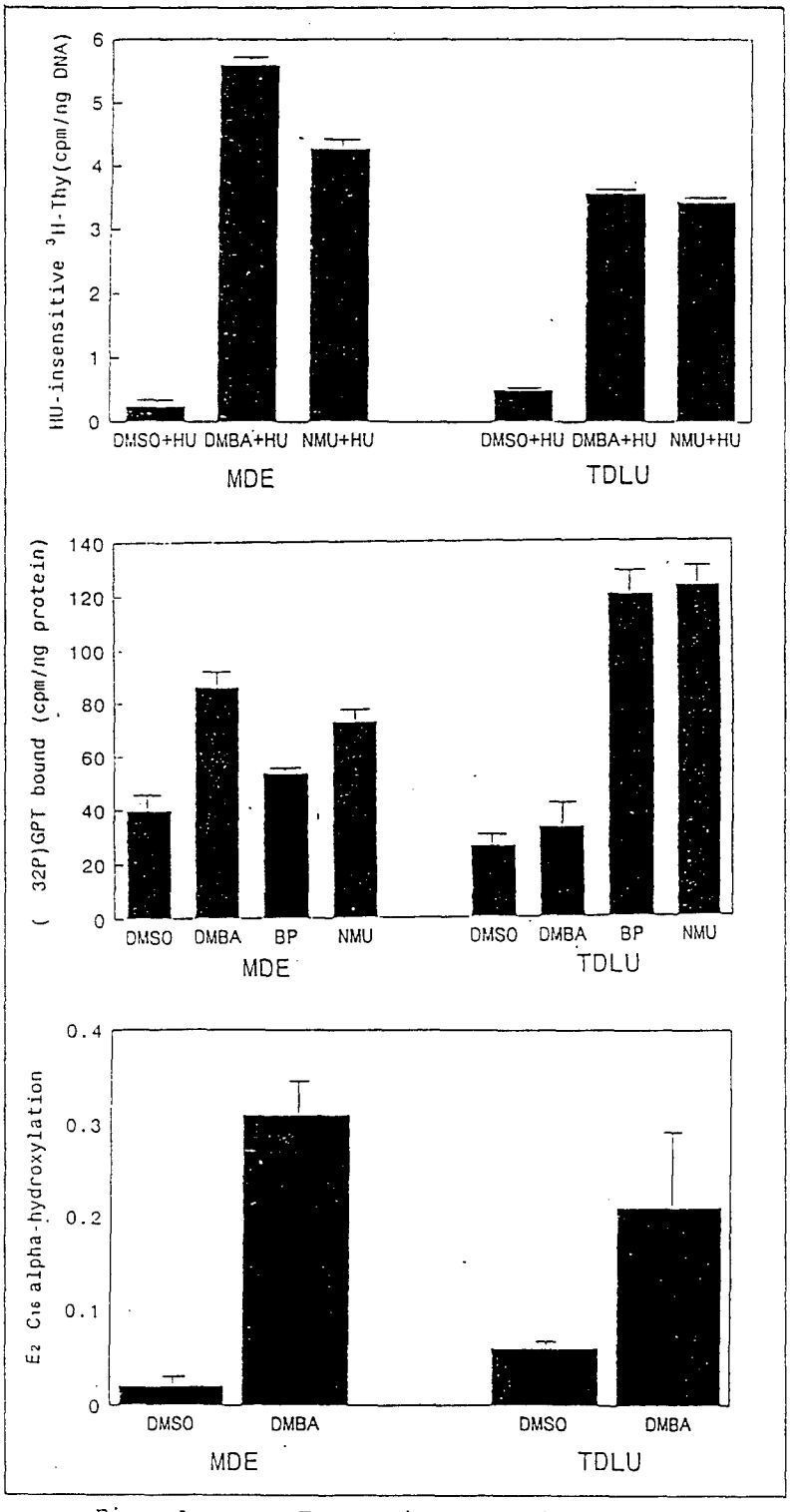

Fig. 1a-c: Expression of biochemical and endocrine markers in murine MDE and human TDLU treated with chemical carcinogens.

increased extent of estradiol C16α-hydroxylation.

Modulation of carcinogenesis: To examine whether the effect of chemical carcinogens can be altered by polyunsaturated fatty acids, carcinogen-initiated MDE and TDLU were treated with LNA or EPA. The data presented (Fig. 2a,b) demonstrated that the tumor promoting LNA enhanced DMBA or BP-induced Ras p21-GTP binding and cellular E_2 C16α-hydroxylation. In contrast, tumor inhibiting EPA was found to be ineffective in upregulation of GTP binding or E_2 metabolism. The underlying mechanisms for the observed modulation of carcinogenesis by fatty acids warrant further investigation. It is,

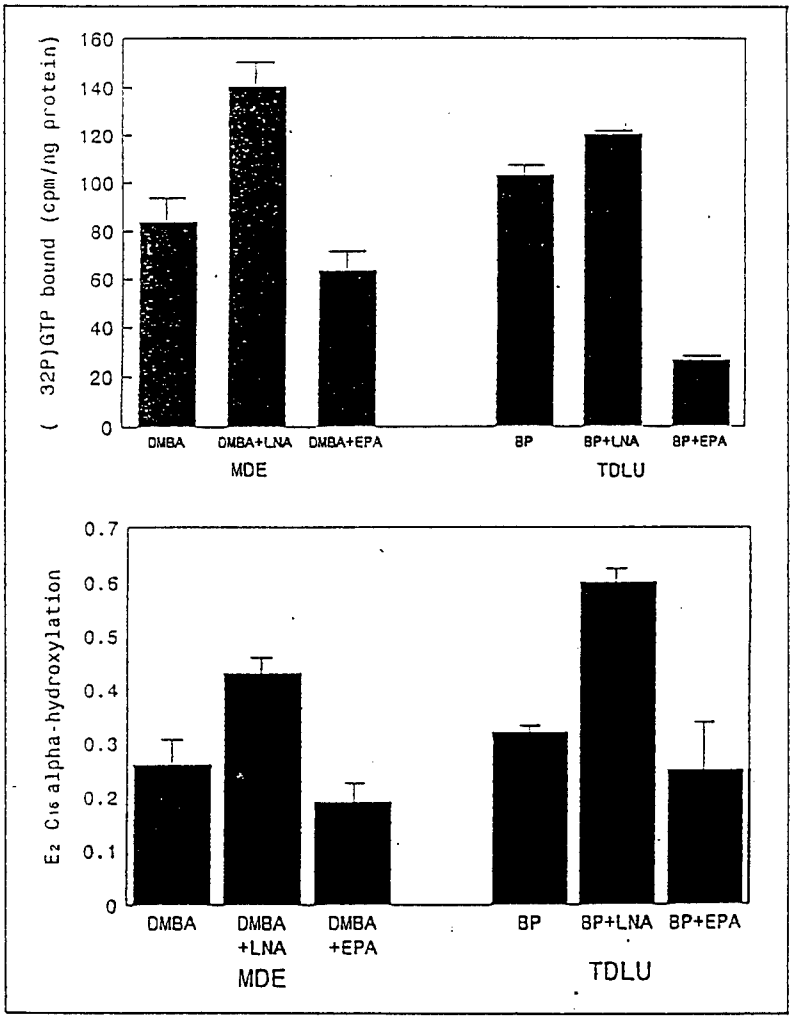

Fig. 1a,b: Modulation of Ras p21-GTP binding and E_2 C16α-hydroxylation in carcinogen treated murine MDE and human TDLU by polyunsaturated fatty acids.

however, noteworthy that the two fatty acids modulate GTPase activity (11), and 16α-hydroxyestrone, the end product of E_2 metabolism is a potent hyperproliferative agent (12).

In conclusion, this study has identified biochemical end points that represent common biomarkers for carcinogen susceptibility of murine and human mammary tissue. These biomarkers provide end points for elevated risk for tumorigenic transformation. Effective modulation of the biomarkers by dietary fatty acids in human tissue suggest their potential clinical relevance for preventive intervention.

ACKNOWLEDGEMENTS

This study is supported in part by NIH grants R29 CA 44741, PO1 CA 29502, grant # DAMD 17-94-J-4208 from the Department of the Army and philanthropic funds to Strang Cancer Prevention Center.

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Proc. Amer. Assoc. Cancer Res. 1995

IN VITRO MODULATION OF HUMAN MAMMARY CARCINOGENESIS: A MODEL FOR CHEMOPREVENTION. Nitin T. TELANG, Division of Carcinogenesis & Prevention, Strang-Cornell Cancer Research Laboratory, Cornell University Medical College, New York, NY/USA

The natural estrogen 17β-estradiol (E_2) influences the mammary cell proliferation, morphogenesis, and neoplastic transformation in vivo. The role of epithelial-stromal interaction in the cellular effects of E_2 is equivocal. Experiments were designed using an in vitro model developed from mammary explants and cell culture to examine whether i.) stromal component affects cellular metabolism of E_2 and ii.) naturally-occurring agents modulate E_2 metabolism. Metabolic conversion of E_2 via C2- and C16α-hydroxylation pathways was monitored by a radiometric assay, and cellular proliferation was measured by ^{3}H-thymidine uptake or by anchorage-independent growth. In the explants of terminal duct lobular units (TDLU) containing inter-lobular and inter-ductal stroma, the chemical carcinogen benzo(a)pyrene (BP) induced a 164.8% increase in E_2 C16α-hydroxylation ($P=0.006$) and a 51.9% increase in ^{3}H-thymidine uptake ($P=0.002$). BP treatment also exhibited a 77.6% decrease ($P<0.0001$) in E_2 C2/C16α-hydroxylation ratio, which was abrogated ($P<0.0001$) in the presence of eicosapentaenoic acid (EPA) or indole-3-carbinol (I3C). In the cultures of human mammary carcinoma MCF-7 cells EPA and I3C enhanced E_2 C2/C16α-hydroxylation ratio ($P<0.0001$) and inhibited anchorage-independent growth ($P=0.001$). In TDLU and MCF-7 cultures the extent of E_2 C2-hydroxylation was down-regulated ($P=0.005$) by human adipocyte-conditioned medium (h-ACM) obtained from obese subjects. Thus, BP- and h-ACM-induced alteration of E_2 metabolism and its modulation by EPA and I3C in TDLU suggests a paracrine role of stroma in human mammary carcinogenesis. This in vitro model may provide a system to assess the effects of naturally-occurring chemopreventive agents on human mammary cell transformation [Support: Dept. of the Army Grant # DAMD17-94-J-4208, NIH PO1 CA 29502 and the Wanda Jablonski Fund].

Breast Cancer Res. Treat. 1995

CHEMOPREVENTION OF HUMAN MAMMARY CARCINOGENESIS BY NATURALLY OCCURRING PHYTOCHEMICALS.

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Overexpression of HER/2-neu oncogene confers tumorigenic transformation in the mammary tissue of transgenic mice, and in immortalized human mammary epithelial cells. This oncogene also represents a prognostic marker for early relapse of human breast cancer. The experiments in the present study were designed to: i) determine the growth kinetics of immortalized, non-tumorigenic human mammary epithelial 184-B5 and HER/2neu oncogene-initiated 184-B5 /HER cells, and ii) demonstrate the response of the two cell lines to selected phytochemicals that function as tumor inhibitors in rodent models.The oncogene initiated 184-B5/HER cells exhibited about 34% shorter population doubling time ($P=0.001$) relative to that of parental 184-B5 cells, suggesting induction of oncogene-mediated aberrant hyperproliferation. A continuous 7-day treatment with (-)epigallocatechin gallate (EGCG,a green tea polyphenol), indole-3-carbinol (I3C,a plant indole) and genistein (GEN,a soy isoflavone) showed differential growth arrest of the two cell lines. Effective inhibitory concentrations of the phytochemicals were at least 3-10 fold higher for 184-B5/HER relative to those for 184-B5 cells.

Thus, oncogene-induced aberrant hyperproliferation may represent a cellular marker for phytochemical-mediated chemoprevention of human mammary carcinogenesis. [Support: Indo-US Fulbright fellowship #17267, Dept. of Army grant #DAMD 17-94-J-4208 and Strang Philanthropic Funds].

Chemoprevention of Mammary Preneoplasia

In Vitro Effects of a Green Tea Polyphenol[a]

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Cornell University Medical College
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Strang Cancer Prevention Center
New York, New York 10021

INTRODUCTION

The multistage development of mammary cancer is characterized by the presence of aberrant hyperproliferation and hyperplasia that precede the appearance of adenocarcinoma.[1–3] In the murine model of mammary carcinogenesis, such lesions as ductal hyperplasia and hyperplastic alveolar nodules are induced in response to treatment with chemical carcinogens,[4] oncogene expression in transgenic mice,[5,6] or transmission of milk-borne murine mammary tumor virus (MTV).[7]

Transplantation of hyperplastic lesions results in a high incidence of carcinoma at the transplant site.[1,3] Recent experiments on spontaneously immortalized, nontumorigenic mammary epithelial cell lines have demonstrated that exposure to chemical carcinogens or transfection with oncogenes results in upregulation of aberrant hyperproliferation *in vitro* and hyperplasia *in vivo* prior to the appearance of adenocarcinoma.[8–10] Aberrant hyperproliferation *in vitro* and hyperplasia *in vivo* may therefore represent a cellular marker for preneoplastic transformation.

The experiments in the present study were designed to (i) examine the extent of preneoplastic and neoplastic transformation in mouse mammary epithelial cells initiated for tumorigenic transformation by deregulated expression of c-myc oncogene or of MTV and (ii) evaluate chemopreventive efficacy of a green tea polyphenol, (−)-epigallocatechin gallate (EGCG), against mammary preneoplastic transformation. EGCG inhibits chemical carcinogen–induced tumor development of several organ sites.[11–15]

[a]This work was supported in part by National Institutes of Health Grant No. P 01 CA 29502, Department of the Army Grant No. DAMD 17-94-J-4208, and philanthropic funds to the Strang Cancer Prevention Center.

MATERIALS AND METHODS

Cell Lines

The mammary epithelial cell lines utilized in the present study are established from the noninvolved mammary tissue of the low-cancer-risk BALB/c and the high-cancer-risk RIII strains of mice. The stable c-myc transfectant MMEC/myc_3 and the MTV-expressing RIII/MG, because of their initiated status, represent the preneoplastic phenotypes. These two cell lines are used in the experiments designed to examine EGCG-mediated modulation of preneoplastic transformation. The tumor-derived cell lines MMEC/myc_3-Pr_1 and RIII/Pr_1 are used as the positive controls to validate aberrant hyperproliferation as the quantitative endpoint for preneoplastic transformation. The cell lines were grown in DME/F12 medium supplemented with 10% fetal bovine serum, 4 mM *l*-glutamine, 5 μg/mL insulin, and antibiotics according to the published procedure.[10]

Anchorage-dependent and Anchorage-independent Growth Assays

The growth assays were performed according to the previously published procedures[8–10] to examine the short-term (4-day duration) and long-term (14-day duration) effects of EGCG on preneoplastic transformation initiated by c-myc or MTV on mammary epithelial cells.

Tumorigenicity Assay

The tumorigenic transformation of AIG-positive MMEC/myc_3 and RIII/MG cells was evaluated *in vivo* by the mammary fat pad transplantation technique.[3,4,10,16] The recipients were palpated at weekly intervals for the presence of tumor.

Treatment with EGCG

The stock solution of EGCG (1000×) was made up in phosphate-buffered saline (PBS, pH 7.2) and was serially diluted with the culture medium to obtain the final concentrations of the compound at 0.1, 0.5, 1.0, and 10 μg/mL.

RESULTS

Effect of EGCG on Anchorage-dependent Growth (ADG)

The dose response of EGCG on ADG of MMEC/myc_3 and RIII/MG cells is presented in FIGURES 1A and 1B. MMEC/myc_3 cells not exposed to EGCG showed almost

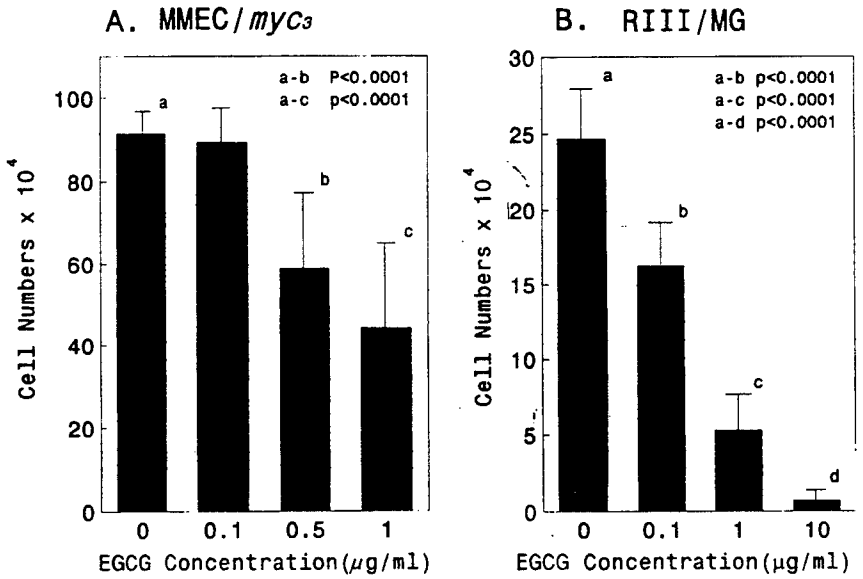

FIGURE 1. Effect of EGCG on anchorage-dependent growth in MMEC/myc$_3$ cells (A) and in RIII/MG cells (B).

a 90-fold increase in 4 days relative to the initial seeding density of 1.0×10^4 cells. Following a continuous treatment with 0.1, 0.5, and 1.0 μg/mL EGCG, MMEC/myc$_3$ cells exhibited a progressive, concentration-dependent decrease in the surviving cell population. Treatment with 0.5 μg/mL and 1.0 μg/mL EGCG exhibited a significant decrease ($P < 0.0001$) in the surviving population, indicating an antiproliferative effect of the polyphenol (FIGURE 1A). The response of RIII/MG cells to EGCG is shown in FIGURE 1B. It was interesting to note that 0.1 μg/mL EGCG, while ineffective in MMEC/myc$_3$ cells, was able to induce about a 40% decrease ($P < 0.0001$) in RIII/MG cells. EGCG at higher doses of 1.0 μg/mL induced a 53.3% and 76.0% inhibition of growth in MMEC/myc$_3$ and RIII/MG cells, respectively.

Effect of EGCG on Anchorage-independent Growth (AIG)

The optimal antiproliferative concentrations of EGCG identified by the ADG assay were examined for their long-term effects using the AIG assay. A continuous 14-day treatment of MMEC/myc$_3$ cells with 1.0 μg/mL EGCG resulted in a 25% inhibition ($P < 0.0001$) in the number of anchorage-independent colonies (FIGURE 2A). In contrast, treatment of RIII/MG cells with 0.1 μg/mL EGCG resulted in an 88.9% inhibition ($P = 0.0001$) in the number of anchorage-independent colonies (FIGURE 2B).

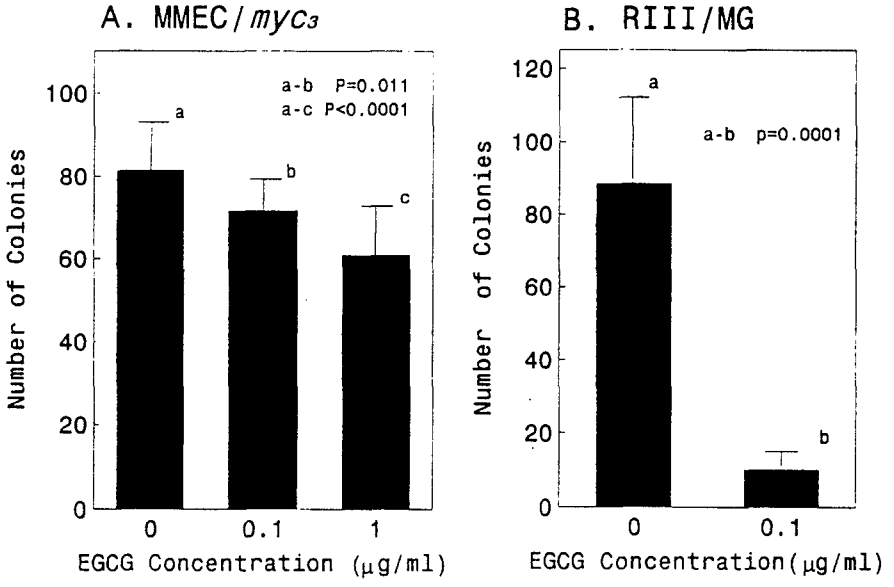

FIGURE 2. Effect of EGCG on anchorage-independent growth in MMEC/myc$_3$ cells (A) and in RIII/MG cells (B).

Tumorigenic Transformation of MMEC/myc$_3$ and RIII/MG Cells

The tumorigenicity of the two initiated cell lines was compared with that of tumor-derived phenotypes MMEC/myc$_3$-Pr$_1$ and RIII/Pr$_1$ representing the positive controls (FIGURES 3A and 3B). The initiated cell lines were able to form rapidly growing tumors at the transplant site that induced a substantial inhibition of disease-free survival.

DISCUSSION

This study has utilized mouse mammary epithelial cell lines initiated by c-myc oncogene and MTV to examine whether a naturally occurring polyphenol present in Japanese green tea modulates the process of preneoplastic transformation. To this end, the relative extent of AIG has been utilized as the major quantitative endpoint. AIG represents a useful *in vitro* biomarker for preneoplastic and neoplastic transformation.[8–10] The data generated from the present study essentially confirm and extend our previous observations and indicate that nontumorigenic mammary epithelial cells, upon

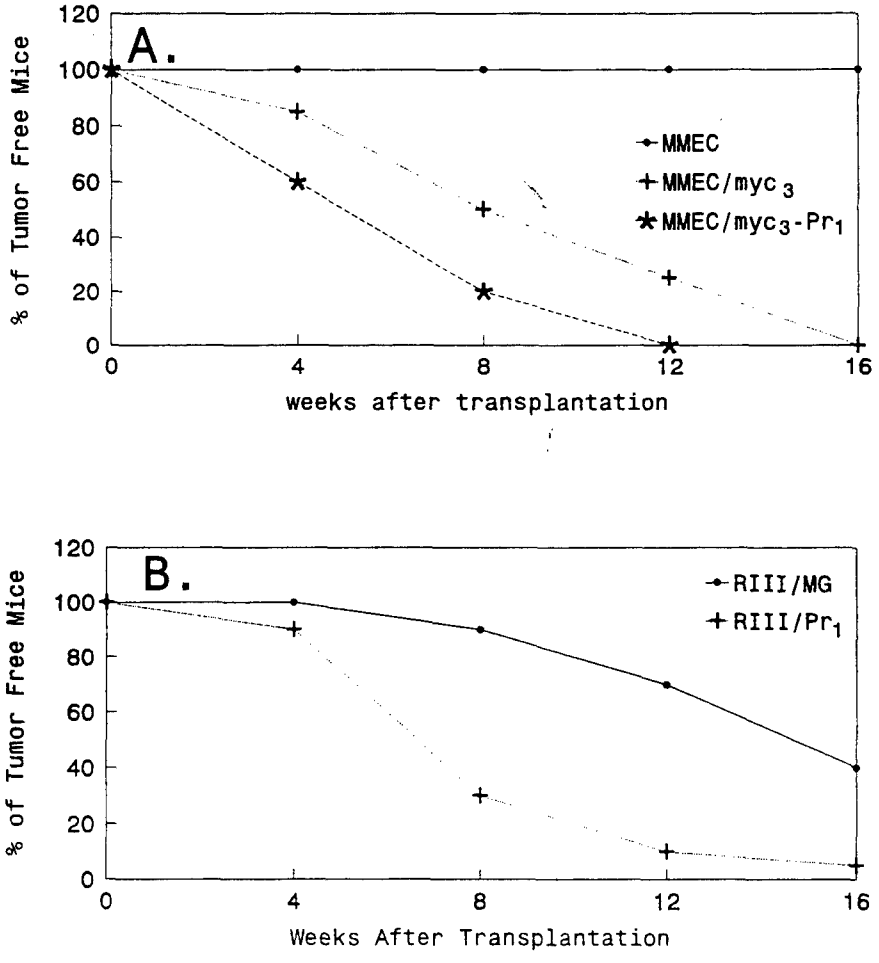

FIGURE 3. Tumorigenicity of MMEC/myc_3 and MMEC/myc_3-Pr_1 cells (A) and of RIII/MG and RIII/Pr_1 cells (B) as seen by a decrease in the % of tumor-free mice.

initiation by c-myc oncogene or MTV, express AIG *in vitro* and tumorigenesis *in vivo*. Furthermore, because AIG is detected in initiated MMEC/myc_3 and RIII/MG cells as well as in tumor-derived MMEC/myc_3-Pr_1 and RIII/Pr_1 cells, upregulation of AIG in cells initiated by c-myc oncogene or by MTV represents a marker for preneoplastic transformation.

Aqueous extract of green tea provides a complex mixture of polyphenols, caffeine, and thrombin. Among the polyphenols, EGCG represents a major water-soluble

fraction of the tea extract.[12–15] The aqueous extract of tea as well as the major polyphenolic component, EGCG, suppress procarcinogen-induced organ-site tumorigenesis *in vivo*. During the multistage development of organ-site cancer, EGCG is reported to influence carcinogen activation and DNA adduct formation,[13] as well as generation of free-radical-mediated oxidative DNA damage,[14] all of which are critical targets for initiation of carcinogenesis. In addition, EGCG is reported to affect the promotional stage of carcinogenesis in part via modulation of P450-dependent enzymes critical for sustained proliferative activity of the initiated phenotype.[17–19] This aspect of the biological activity of EGCG appears to be more relevant to the present study because AIG represents a late-occurring, postinitiational (promotional) event in the multistage process of carcinogenesis. The experiments designed to examine the effects of EGCG on initiated MMEC/myc$_3$ and RIII/MG cells revealed a dose-dependent suppression of proliferation in the anchorage-dependent condition of growth. It was also noteworthy that the optimal antiproliferative dose of EGCG differed by at least 10-fold in the two cell lines. The oncogene-initiated cells required a 10-fold higher dose of EGCG (1.0 μg/mL) than the virus-initiated cells (0.1 μg/mL) to suppress AIG. This differential responsiveness may be attributed to the type of initiator. In this context, it is notable that deregulated expression of c-myc oncogene in transgenic mice results in early onset of mammary adenocarcinomas,[5,6] whereas MTV is considered a weakly transforming retrovirus that produces slow-growing mammary adenocarcinomas *in vivo*.[7] These observations, together with our data on the differential responsiveness of MMEC/myc$_3$ and RIII/MG cells to EGCG, raise the possibility that the potency of the oncogene being higher than that of MTV as an initiator leading to a greater extent of transformation requires a higher concentration of EGCG for its preventive efficacy. Clearly, this aspect needs to be systematically analyzed by mechanism-oriented experiments.

SUMMARY

The major findings of this study can be summarized as follows:

(1) the c-myc oncogene-transfected and MTV-expressing mammary epithelial cells exhibit aberrant hyperproliferation *in vitro* preceding tumorigenesis *in vivo*;
(2) upregulation of aberrant hyperproliferation (i.e., anchorage-independent growth) in initiated cells represents a cellular marker for preneoplastic transformation;
(3) the tea polyphenol EGCG differentially downregulates aberrant hyperproliferation in myc oncogene- and MTV-initiated cells;
(4) the present *in vitro* model provides an efficient assay for chemoprevention of mammary preneoplasia by naturally occurring compounds.

ACKNOWLEDGMENTS

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The Role of Estrogen in Mammary Carcinogenesis[a]

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INTRODUCTION

Human breast cancer is one of the most prevalent diseases in women. The American Cancer Society estimates approximately 182,000 new cases of breast cancer and about 46,000 breast cancer–related deaths for 1994.[1] The epidemiological evidence provides strong support for the concept that environmental, hormonal, and genetic factors affect the risk for development of breast cancer.[2–6] From among the various recognized risk factors, age at menarche, age at first pregnancy, and age at menopause suggest that endogenous ovarian steroids may profoundly affect initiation, promotion, and progression of carcinogenesis.[3,6,7]

Experimental studies on rodent mammary tissue have demonstrated that the ovarian steroids, 17β-estradiol (E_2) and progesterone (Prg), acting in concert, induce proliferation and positively regulate mammary epithelial morphogenesis, that is, formation of the epithelial ductal system exhibiting proliferative terminal end-buds (TEB). In response to such mammotropic/lactogenic hormones as adrenal glucocorticoids and the pituitary polypeptide prolactin, TEB are induced for functional cytodifferentiation as evidenced by the presence of transformation to secretory lobulo-alveoli. The selected steroid and polypeptide hormones that are mammotropic in the rodent mammary system are also reported to regulate epithelial cell proliferation in human mammary terminal duct lobular units (TDLU), the principal target site for carcinogenesis in humans.[5,7,8]

From among the mammotropic hormones as endogenous factors that influence mammary carcinogenesis, estrogens have attracted the most attention. The natural estrogen E_2 is a well-known promoter of rodent mammary carcinogenesis. Carcinogen-induced as well as spontaneous mammary tumors in rodent models are negatively growth-regulated by surgical or chemical ablation of ovarian function.[2,3,5–7,9,10] E_2 induces DNA synthesis in quiescent cells, increases the expression of oncogenes, and functions as a potent mitogen in estrogen-responsive tissues.[3,6,11] Taken together, these observations provide evidence that estrogens, because of their mitogenic property, may increase the susceptibility of the target tissue to initiation. Additionally, E_2-mediated mitogenicity on preinitiated target cells may predispose these cells to tumorigenic transformation.

Microsomal hydroxysteroid dehydrogenases and P450-dependent steroid hydroxy-

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lases are critical for the estrogen biotransformation that converts highly estrogenic E_2 to progressively less estrogenic metabolites.[12,13]

In vivo experiments utilizing strains of mice that differ in their relative risk of developing the murine mammary tumor virus (MTV)–induced mammary adenocarcinoma demonstrated that the relative extent of metabolism of E_2 via the C16α-hydroxylation pathway is higher in MTV-expressing, high mammary cancer strains of mice such as C3H and RIII relative to those strains such as BALB/c and C57 that do not express MTV, and as a result exhibit low incidence of mammary adenocarcinoma. More importantly, the upregulation of the E_2 C16α-hydroxylation pathway was detectable in (6–8)-week-old virgin female mice well before the appearance of mammary adenocarcinoma.[14] The clinical relevance of the status of E_2 metabolism as an endocrine biomarker for human mammary carcinogenesis was suggested by investigations on breast cancer patients and subjects at risk for breast cancer.[14–16] These studies revealed that E_2 metabolism via the C17-oxidation and C2-hydroxylation pathways was essentially unaltered, whereas the relative extent of E_2 metabolism via the C16α-hydroxylation pathway was selectively and specifically increased in subjects at risk and in patients with identifiable breast cancer. Taken together, these *in vivo* studies suggested that upregulation of E_2 C16α-hydroxylation is associated with increased risk for breast cancer or with the presence of breast cancer, and therefore may represent a useful endocrine biomarker for mammary carcinogenesis. The *in vivo* experiments were conducted on strains of mice that were "initiated" because of MTV expression. The clinical studies utilized either subjects at risk or patients with breast cancer, where the target organ was either "initiated" or "transformed". It is therefore conceivable that upregulated E_2 C16α-hydroxylation may represent a biomarker for the tumor-promoting effect of E_2. Furthermore, the *in vivo* studies discussed above provide evidence for systemic metabolism of E_2 where such nontarget tissues for breast cancer as liver, lung, and adrenals represent the major sites of E_2 metabolism. These experiments, therefore, provide little information with regard to the extent of E_2 metabolism at the target site for breast cancer, that is, the mammary tissue.

To examine whether the natural estrogen E_2 functions as an initiator or promoter of mammary carcinogenesis, several *in vitro* studies have been conducted utilizing rodent and human mammary explant culture and cell culture systems.[17–22] The present report attempts to provide a brief overview of some of the recent experiments that were designed to understand the role of estrogens in mammary carcinogenesis.

EXPERIMENTAL SYSTEMS

Rodent and Human Mammary Explant Culture Studies

The mammary explant culture model provides a useful system for examining the responsiveness of the transformation-sensitive target tissue directly to agents that influence mammary cell proliferation, cytodifferentiation, and neoplastic transformation.[17–22] We have pursued this approach to evaluate the ability of mammary tissue to metabolize E_2 and to examine whether the extent of metabolism correlates with mammary carcinogenesis.

The relative extent of E_2 metabolism via the C16α-hydroxylation pathway was

TABLE 1. Metabolism of 17β-Estradiol (E_2) in Explant Cultures of Mammary Tissue and Liver from Mice at Differing Risk for Breast Cancer

Mouse Strain	Cancer Risk	Tissue Type	E_2 Metabolism (% per mg tissue)	
			C17-Oxidation	C16α-Hydroxylation
NFS	low	liver	0.72 ± 0.41	0.31 ± 0.17
		mammary	0.20 ± 0.03[a]	0.03 ± 0.02[c]
C3H/OuJ	high	liver	2.38 ± 1.36	0.38 ± 0.17
		mammary	0.59 ± 0.13[b]	0.13 ± 0.03[d]

[a–d]Mean ± SD, $n = 18$; *a-b*, *c-d*: $p \leq 0.001$.

determined in the explant cultures of liver tissue and of mammary tissue from NFS (low breast cancer risk) and C3H/OuJ (high breast cancer risk) strains of mice. The extent of E_2 C16α-hydroxylation was comparable in the liver tissue from the two strains, whereas it was increased by 4-fold ($p = 0.001$) in the mammary tissue from C3H/OuJ mice relative to that from NFS mice.[20] The specific and significant increase in E_2 C16α-hydroxylation in the mammary tissue suggests a cancer risk–dependent modulation of E_2 metabolism in the target tissue for mammary carcinogenesis (TABLE 1).

To examine whether E_2 metabolism is altered in response to exposure to chemical carcinogens, mammary explant cultures from the BALB/c (low breast cancer risk) mouse strain were treated with the chemical carcinogen, 7,12-dimethylbenz(a)anthracene (DMBA), and the extent of E_2 C16α-hydroxylation was determined.[17] Treatment of explant cultures with 3.9 μM and 7.8 μM DMBA resulted in a 6.8-fold increase ($p < 0.02$) and a 15.1-fold increase ($p < 0.0001$) in E_2 C16α-hydroxylation, respectively, relative to that in controls (TABLE 2).

In an effort to extend the clinical relevance of altered E_2 metabolism, experiments on human mammary explant cultures were performed.[18,19] In our study, the extent of E_2 metabolism via the C17-oxidation, C2-hydroxylation, and C16α-hydroxylation pathways was compared in the explant cultures of human mammary terminal duct lobular units (TDLU) obtained from patients undergoing surgery for breast cancer. This study revealed that noninvolved TDLU, the presumptive site for human mammary carcinogenesis, effectively metabolized E_2 via the major metabolic pathways operative *in vivo*.

TABLE 2. Effect of 7,12-Dimethylbenz(a)anthracene (DMBA) on 17β-Estradiol (E_2) Metabolism in Mouse Mammary Explant Cultures

Treatment	E_2 Metabolism ($C16\alpha/C_2$ ratio)[a]	16α-OHE_1 Formed (nmoles/mg tissue)[b]
0.1% DMSO (solvent control)	0.49 ± 0.06[c]	0.32 ± 0.10[f]
3.9 μM	0.86 ± 0.08[d]	2.50 ± 0.43[g]
7.8 μM	1.89 ± 0.10[e]	5.16 ± 0.30[h]

[a]Amount of ^3H_2O formed after a 48-h incubation with [C2-^{3}H]E_2 and [C16α-^{3}H]E_2.
[b]Calculated from the specific activity of [C16α-^{3}H]E_2.
[c–h]Mean ± SD, $n = 6$; *c-d*: $p = 0.003$; *c-e*: $p = 0.0001$; *f-g*, *f-h*: $p = 0.0001$.

TABLE 3. Metabolism of 17β-Estradiol (E_2) by Explant Cultures of Human Mammary Terminal Duct Lobular Units

Duration of Exposure to $[^3H]E_2$	E_2 Metabolism (% per mg tissue)[a]		
	C17-Oxidation	C2-Hydroxylation	C16α-Hydroxylation
24 h	0.163 ± 0.011[b]	0.079 ± 0.004[d]	0.009 ± 0.002[f]
72 h	0.314 ± 0.038[c]	0.203 ± 0.039[e]	0.071 ± 0.032[g]

[a]Amount of ^3H_2O formed after incubation with specifically labeled E_2.
[b–g]Mean ± SD, $n = 6$; *b-c*: $p < 0.002$; *d-e*: $p < 0.07$; *f-g*: $p < 0.001$.

It was of interest to note that the relative extent of C17-oxidation and of C16α-hydroxylation exhibited a significant time-dependent increase, whereas C2-hydroxylation remained essentially unaltered with respect to the time of incubation with $[C2\text{-}^3H]E_2$. These observations raise the possibility that, in TDLU from cancerous breast tissue, estrone formed by C17-oxidation of E_2 is preferentially converted to 16α-hydroxyestrone via the C16α-hydroxylation pathway (TABLE 3). In this context, it is noteworthy that the relative extent of E_2 C16α-hydroxylation increased correspondingly with the phase of the menstrual cycle. TDLU obtained from patients in the luteal phase exhibited about a 4-fold increase in E_2 C16α-hydroxylation relative to those from patients in their follicular phase (TABLE 4). The observed differences in the E_2 C16α-hydroxylation pathway during luteal and follicular phases of the menstrual cycle raise the possibility that altered E_2 metabolism may reflect tissue response to serum levels of E_2, which functions as the substrate for the metabolic reactions.[3,6,7,23]

More recently, the human mammary TDLU explant culture model has been utilized to examine whether the extent of E_2 C16α-hydroxylation is related to the risk of developing breast cancer.[21,22] The relative extent of C16α-hydroxylation of E_2 was determined in explant cultures of mammary fat (MF, nontarget tissue for breast cancer) and in TDLU (target tissue for breast cancer). The two types of noninvolved tissues were obtained from reduction mammoplasty specimens (low risk for cancer) and from patients undergoing surgery for breast cancer (high risk for cancer). The relative extent of E_2

TABLE 4. Influence of the Phase of Menstrual Cycle on Estradiol C16α-Hydroxylation in Explant Cultures of Human Mammary Tissue

Phase of Menstrual Cycle	Type of Tissue	Extent of E_2 C16α-Hydroxylation (% per mg tissue)[a]	Relative Increase[b]
Follicular (day 1–15)	MF	0.051 ± 0.008[c]	—
	TDLU	0.178 ± 0.060[d]	2.49
Luteal (day 16–1)	MF	0.029 ± 0.006[e]	—
	TDLU	0.935 ± 0.040[f]	31.24

[a]Amount of ^3H_2O formed after a 48-h incubation with $[C16\alpha\text{-}^3H]E_2$.
[b](TDLU − MF)/MF.
[c–f]Mean ± SD, $n = 6$; *c-d*, *e-f*: $p = 0.001$.

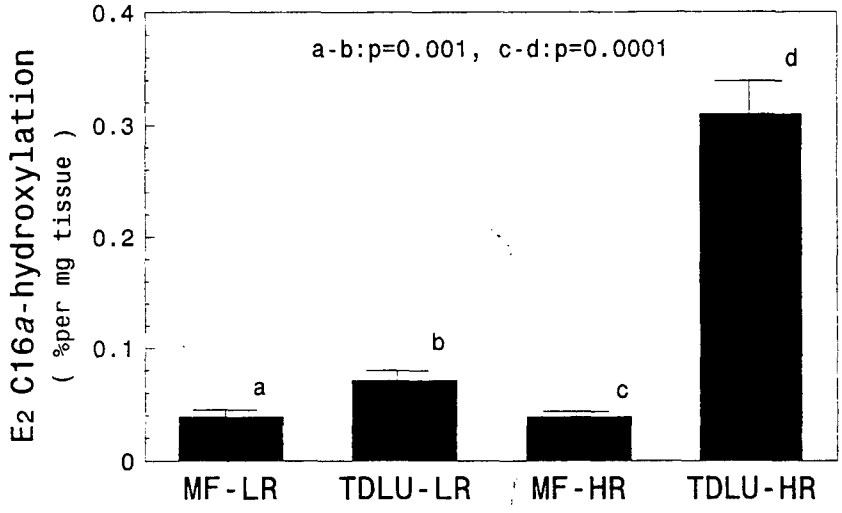

FIGURE 1. Breast cancer risk–dependent alteration of 17β-estradiol metabolism in explant cultures of human mammary tissue.

C16α-hydroxylation was 82% higher in low-risk (LR) TDLU and was 695% higher in high-risk (HR) TDLU than that observed in corresponding mammary fat. Furthermore, high-risk TDLU exhibited a 337% increase in E_2 C16α-hydroxylation relative to that observed in low-risk TDLU (FIGURE 1).

Having demonstrated that constitutive levels of E_2 C16α-hydroxylation are altered in human mammary TDLU explant culture dependent upon the relative risk for developing breast cancer, it was of interest to examine whether exposure to chemical carcinogens results in alteration of E_2 metabolism. Low-risk and high-risk TDLU were treated with the chemical carcinogen, benzo(α)pyrene (BP), and were utilized to determine the extent of E_2 C16α-hydroxylation. It was observed that, in low-risk TDLU, BP treatment resulted in a 57.4% increase ($p < 0.01$) in the extent of E_2 C16α-hydroxylation relative to that seen in the solvent controls. In contrast, in high-risk TDLU, BP treatment induced a 246.1% increase ($p < 0.001$) relative to that seen in solvent controls.[21] Taken together, the studies conducted on the human mammary TDLU raise the possibility that breast cancer risk–dependent differences in constitutive and carcinogen-induced levels of E_2 16α-hydroxylation may be a manifestation of intrinsic hyperresponsiveness of high-risk TDLU (FIGURE 2).

Having demonstrated that E_2 C16α-hydroxylation is upregulated in rodent as well as in human mammary explant cultures in response to known initiators of carcinogenesis such as chemical carcinogens and transforming retrovirus, it was of interest to examine whether this endocrine responsiveness of the target tissue can be altered by agents that are known to modulate rodent mammary carcinogenesis. It is well documented that diets rich in polyunsaturated omega-6 fatty acids (n-6 PUFA) function as tumor promoters, whereas diets rich in polyunsaturated omega-3 fatty acids (n-3 PUFA) inhibit tumor growth in the chemical carcinogen–induced or spontaneous rodent mammary tumor

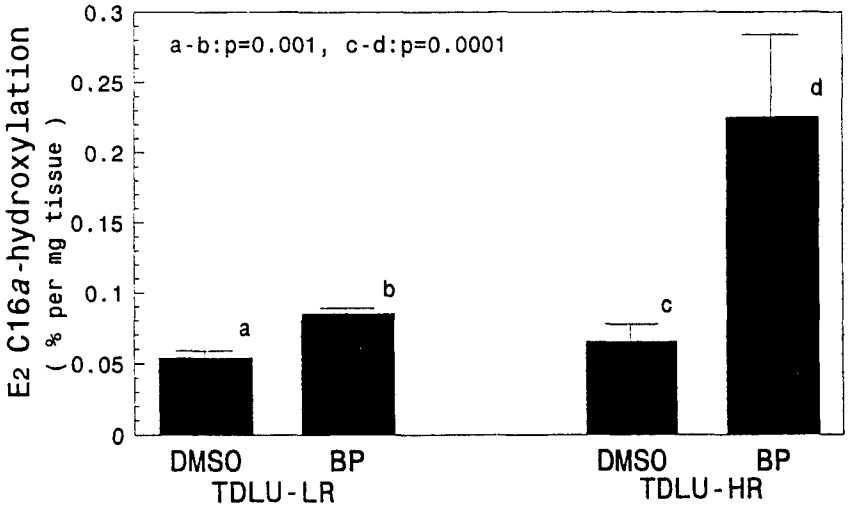

FIGURE 2. Effect of benzo(α)pyrene (BP) on estradiol metabolism in explant cultures of human mammary terminal duct lobular units.

model.[6,9,24–28] Linoleic acid (LNA) represents a major component of the tumor-promoting diet, whereas eicosapentaenoic acid (EPA) is the prevalent component of the diet that suppresses tumor growth.

In one experiment where the process of tumorigenic transformation was initiated with the chemical carcinogen DMBA in rodent explant cultures, the presence of n-6 PUFA was found to enhance the E_2 C16α-hydroxylation pathway by about 66% relative to that observed in cultures treated only with DMBA ($p = 0.014$). In contrast, the presence of n-3 PUFA induced about a 43% decrease ($p = 0.005$) in the E_2 C16α-hydroxylation pathway.[17] These results suggest that the ability of dietary PUFA to modulate mammary carcinogenesis may be due in part to alteration in E_2 C16α-hydroxylation (FIGURE 3, left panel).

To examine whether human mammary tissue is responsive to n-6 and n-3 PUFA, constitutive levels of E_2 C16α-hydroxylation were measured in low-risk and high-risk TDLU maintained in the presence of LNA and EPA. Treatment with LNA increased, whereas that with EPA decreased the relative extent of E_2 C16α-hydroxylation (data not shown). The modulatory influence of n-6 and n-3 PUFA on TDLU initiated with the chemical carcinogen BP is presented in the right panel of FIGURE 3. Similar to their effect on DMBA-initiated mouse mammary tissue, n-6 PUFA exhibited a 117% increase in E_2 C16α-hydroxylation ($p = 0.0001$). In contrast, n-3 PUFA lacked the ability to modulate cellular metabolism of E_2 via the C16α-hydroxylation pathway.

Rodent and Human Cell Culture Studies

The experiments using the mammary explant culture system have provided important information with regard to the extent of E_2 metabolism, effects of initiators, and

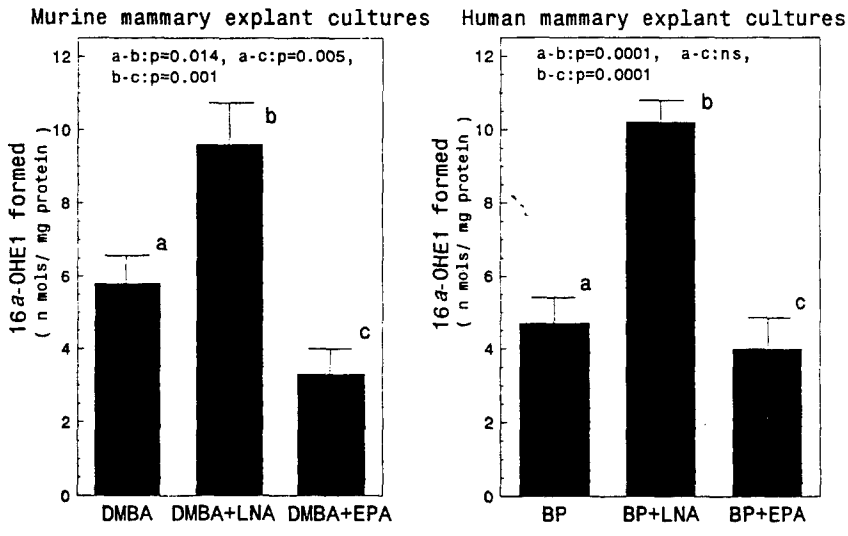

FIGURE 3. Effect of polyunsaturated fatty acids on chemical carcinogen–induced 16α-hydroxyestrone (16α-OHE1) formation in mammary explant cultures.

responsiveness to dietary modulators of tumorigenesis at the target tissue level. The cellular heterogeneity intrinsic to the explant culture system compromises the specificity of experiments designed to examine the direct effects of initiators and modulators of carcinogenesis on the transformation-sensitive epithelial cell. To enhance the sensitivity and specificity of the *in vitro* approach, studies were undertaken on epithelial cell culture systems established from rodent and human mammary tissue and tumors.

In the experiment designed to examine whether E_2 or its oxidative metabolites function as initiator(s) of carcinogenesis, spontaneously immortalized, nontumorigenic murine C57/MG cells were used. The extent of initiation of carcinogenesis was evaluated by determining genotoxicity (unscheduled DNA synthesis, UDS) and anchorage-independent growth (AIG). The cells treated with the prototypic initiator DMBA represented the positive controls. This study revealed that the estrogen metabolite 16α-OHE_1 and DMBA were the most potent inducers of UDS and AIG, whereas E_2 and E_3 were negative in the two assays[29] (FIGURE 4, left and right panels).

The cell culture model to examine the effect of E_2 on human mammary carcinogenesis utilizes the well-characterized human mammary carcinoma cell line MCF-7. This cell line expresses the estrogen and progesterone receptors and produces tumors *in vivo* as evaluated by the nude mouse tumorigenesis assay.

The experiments designed to compare the relative binding affinity of E_2 and/or its metabolites to the estrogen receptor (ER) revealed that 16α-OHE_1, the product of E_2 C16α-hydroxylation, has a weaker binding affinity to ER as well as to the sex hormone binding globulin (SHBG) relative to that of E_2. However, in comparison with other estrogens, the binding affinity of 16α-OHE_1 to ER is irreversible because of a stabilized Schiff base followed by a Heinz rearrangement.[14,30] This was further substantiated by the

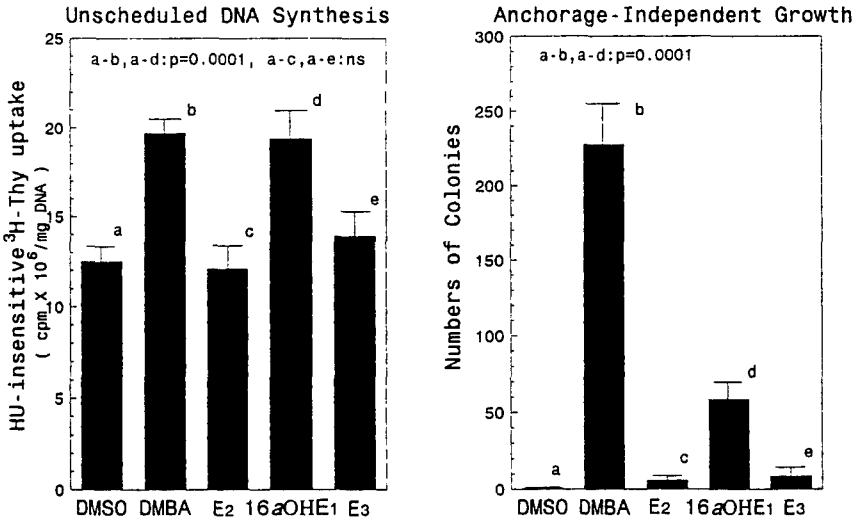

FIGURE 4. Induction of biochemical and cellular markers of carcinogenesis by estradiol metabolites in C57/MG cells.

evidence of covalent binding of labeled 16α-OHE_1 to ER and by immunochemical localization of the covalently bound 16α-OHE_1 to the nuclear matrix.[30]

The impact of binding of 16α-OHE_1 to ER function was examined on the expression of several estrogen-responsive genes such as the progesterone receptor and c-fos, c-jun, and c-myc gene products. It was observed that 16α-OHE_1 downregulates the progesterone receptor and upregulates the expression of the early response genes.[31]

The long-term effect of 16α-OHE_1 on MCF-7 cultures was analyzed by determining the persistent aberrant proliferation and c-myc expression after withdrawal of 16α-OHE_1. It was observed that treatment of MCF-7 cells with 16α-OHE_1 resulted in a long-lasting effect, as evidenced by persistent hyperproliferation and upregulated expression of the c-myc oncogene.[30,31]

SUMMARY OF RESULTS/CONCLUSIONS

The *in vivo* and *in vitro* studies conducted to examine whether E_2 functions as an initiator or a promoter in mammary carcinogenesis can be summarized as follows:

(1) Clinical and animal studies *in vivo* have shown a positive correlation of upregulation of E_2 C16α-hydroxylation with either the presence of or the risk for breast cancer, suggesting that this metabolic alteration may represent an early-occurring event in the multistep process of tumorigenesis.

(2) The mammary tissue, target for carcinogenesis, exhibits cancer risk–dependent alteration in E_2 metabolism in the rodent and human mammary explant culture model, indicating that E_2 metabolites may directly influence the mammary epithelium.

(3) The 16α-hydroxylated metabolite of E_2, 16α-OHE_1, induces genotoxic DNA damage and aberrant hyperproliferation similar to that induced by chemical carcinogens in the rodent cell culture model. In preinitiated or fully transformed rodent or human cells, 16α-OHE_1 promotes the expression of transformed phenotype.

(4) The initiator-mediated perturbation of E_2 C16α-hydroxylation in rodent and human mammary explant cultures is modulated by naturally occurring dietary constituents that are known to modulate rodent mammary tumorigenesis.

(5) The observed effect of E_2 on mammary tumorigenesis may be due in part to the generation of 16α-OHE_1, which functions as a weak initiator or a potent promoter of tumorigenic transformation in mammary epithelial cells.

(6) The reaction of 16α-OHE_1 with the transcription factor ER is unique in that it can be irreversible and leads to aberrant gene expression.

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Oncogenes, Estradiol Biotransformation, and Mammary Carcinogenesis[a]

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INTRODUCTION

The tumorigenic transformation of mammary epithelial cells is the culmination of a multistage process that involves sequential events of initiation, promotion, and progression at genetic and epigenetic levels. This concept is supported by experimental evidence demonstrating that such prototypic initiators as chemical carcinogens, oncogenes, and transforming retrovirus induce mutagenic perturbation (initiation), aberrant hyperproliferation (promotion), and tumorigenesis (progression) in the mammary gland.[1-4] Early-occurring events of initiation and promotion preceding the appearance of adenocarcinoma provide specific and sensitive biomarkers not only for risk identification, but also for efficacious chemoprevention.[5,6]

Oncogenes are recognized positive regulators of growth whose gain of function via point mutation, amplification, overexpression, or translocation is associated with tumorigenic transformation.[7] Chemical carcinogens induce point mutations in the ras protooncogene that are detectable in the initiated mammary tissue as well as in resultant mammary tumors.[8,9] Transgenic mouse models expressing Ras, myc, wnt, and HER-2/neu oncogenes exhibit a high incidence of mammary hyperplasia and adenocarcinoma.[10-15] Thus, cellular protooncogenes may represent molecular targets for initiators of mammary carcinogenesis.

In the endocrine-responsive mammary tissue, the natural estrogen 17β-estradiol (E_2) promotes epithelial cell proliferation and neoplastic transformation, in part due to the mitogenic stimulus via upregulation of such early response genes as c-jun, c-fos, and c-myc.[7,10,11] This mitogenic stimulus to the nontransformed epithelial cell may predispose it to initiation and, in preinitiated cells, the E_2-mediated mitogenic stimulus may promote the expression of the transformed cell phenotype.[5,6] The evidence for E_2 to function as an initiator for mammary carcinogenesis, however, is equivocal. Estrogen receptor (ER) status of the target cell plays an important role in E_2-mediated cellular proliferation and, as such, ER-positivity represents a predictor of therapeutic response to antiestrogens.[3,4,7]

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Cellular biotransformation of E_2 represents a process by which the metabolically competent target cell sequentially converts the mitogenic estrogen E_2 to less-active metabolites. This metabolism of E_2 involves cellular 17β-hydroxysteroid oxidoreductase type 1 and type 2, which are responsible for interconversion of E_2 to estrone (E_1). E_2 and E_1, thus formed, function as substrates for P450-dependent hydroxylases that generate a series of intermediate metabolites capable of exerting pleiotropic agonistic or antagonistic effects on the target cells.[6,16,17] Several *in vivo* studies involving breast cancer patients and animal models have provided evidence that upregulation of E_2C16α-hydroxylation leading to the formation of 16α-hydroxyestrone (16α-OHE_1) correlates with either the risk for or presence of breast cancer.[16–20] Similarly, *in vitro* studies conducted on rodent and human mammary explants or cell cultures have demonstrated that intrinsic metabolism of E_2 is modifiable by selected initiators and modulators of carcinogenesis and that specific E_2 metabolites modulate cell proliferation.[21,22,31] These *in vitro* studies provide evidence that E_2 and/or its metabolites may directly affect the pathophysiology of mammary epithelial cells.

In the multistep process of mammary tumorigenic transformation, early-occurring events of initiation and promotion represent important intermediate steps leading to preneoplastic transformation. At the cellular level, atypical ductal hyperplasia and hyperplastic alveolar nodules represent *in vivo* preneoplastic lesions that are at elevated risk for developing cancer.[1–4,8,10–12] Such lesions are also induced *in vitro* in noninvolved mammary tissue that either is treated with chemical carcinogens or exhibits expression of oncogenes and transforming retrovirus.[23–28] Thus, in the *in vitro* explant culture and cell culture models, aberrant hyperproliferation provides a biomarker for preneoplastic transformation.[5,6,23–31]

Our recent studies on immortalized, nontumorigenic mammary epithelial cell lines have demonstrated that chemical carcinogens and oncogenes induce tumorigenic transformation[25,26,30,31] and that E_2 metabolites influence induction or modulation of preneoplastic transformation.[29,31] These *in vitro* studies provide evidence for a potential link between oncogene expression, biotransformation of E_2, and mammary cell transformation. Such a correlation therefore emphasizes a need for a systematic analysis of the effects of E_2 and its metabolites on oncogene-mediated initiation and promotion of mammary carcinogenesis.

The present report provides an overview of our currently ongoing research program focused on examining oncogene-induced mammary preneoplastic transformation at molecular, endocrine, and cellular levels in an *in vitro* model and on applying the model in an effort to validate the perturbed biomarkers as endpoints for efficacious chemopreventive intervention.

EXPERIMENTAL SYSTEMS

Mammary epithelial cell lines: The parental cell lines C57/MG and MMEC were established from the noninvolved mammary tissue of 6–8-week-old virgin female C57BL/6J and BALB/c mice, respectively.[25,26,29] The two strains of mice exhibit a low incidence of murine mammary tumor virus (MTV)–induced mammary adenocarcinoma.[1,2,4,32]

Oncogene transfection: The parental cell lines C57/MG and MMEC were transfected by electroporation with the plasmids containing v-ras, human c-ras, and human c-myc oncogenes and the gene for resistance to the aminoglycoside antibiotic Genetecin (G418) as the selectable marker. Stable transfectants $C57^{Ras}$,

MMEC/pH06T, and MMEC/myc_3 were obtained by selection and expansion in 400 μg/mL G418.

Neoplastic transformation: The extent of tumorigenic transformation was evaluated by transplantation of the stable transfectants into the mammary fat pads of athymic "nude" mice.[23–25] Resulting adenocarcinomas were reestablished in culture and selected in 400 μg/mL G418. The tumor-derived cell lines MMEC/T_1Pr_1 and MMEC/myc_3Pr_1 were used as the positive controls to validate the relevance of biomarkes as surrogate endpoints for tumorigenic transformation.

Biomarkers for preneoplastic transformation: The endpoints for molecular, endocrine, and cellular biomarkers included Northern blot analysis for oncogene-specific RNA expression,[25,26] E_2 metabolism using radiometric and reverse-phase high-pressure liquid chromatography (RP-HPLC) assays, and aberrant hyperproliferation using an anchorage-independent growth (AIG) assay,[25,26,29–31,33] respectively.

Northern blot analysis utilized [^{32}P]-labeled probes specific for 1.2-kb Ras transcript and 2.8-kb second exon–specific myc transcript, respectively. The extent of E_2 metabolism via C17-oxidation/reduction pathways was determined by product isolation and RP-HPLC, while the extent of E_2 metabolism via C2- and C16α-hydroxylation pathways was determined by measuring ^3H_2O formation after incubation of cell cultures with [C2-^{3}H]E_2 and [C16α-^{3}H]E_2, respectively. The radiometric assay measured the stoichiometric conversion of specifically labeled E_2 to form the intermediate metabolites 2-hydroxyestrone (2-OHE_1) via the C2-hydroxylation pathway and 16α-OHE_1 via the C16α-hydroxylation pathway, respectively.[16–21,34] The AIG assay for aberrant hyperproliferation measured the colony-forming efficiency in 0.33% agar.

Tumor inhibitors: The stock solutions of tamoxifen (TAM), 4-hydroxytamoxifen (4-OH-TAM), indole-3-carbinol (I3C), and eicosapentaenoic acid (EPA) at 1000× were made up in 100% ethanol. The stock solutions were appropriately diluted with the culture medium to obtain the nontoxic concentrations of 1 μM TAM and 4-OH-TAM, 50 μM I3C, and 16 μM EPA, respectively. All the agents at final concentrations exhibited less than 10% reduction in cell number relative to that observed in the controls, as determined by the trypan blue exclusion test and the anchorage-dependent growth assay for viable cell population.[25,26,29–31]

RESULTS AND DISCUSSION

Status of oncogene expression: The initial experiments on the stable transfectants were designed to examine the persistence of oncogene expression and the extent of preneoplastic and neoplastic transformation. The extent of perturbation in Ras and myc oncogene expression *in vitro* (molecular marker) and in aberrant hyperplasia and tumorigenicity *in vivo* (cellular marker) lended support to the concept that deregulated expression of oncogenes represents the initiating event that leads to the tumorigenic transformation of mammary epithelial cells. The parental nontransfected C57/MG and MMEC cell lines represented the negative controls, while tumor-derived MMEC/T_1Pr_1 and MMEC/myc_1Pr_1 represented the positive controls in these experiments. The extent of expression of the 1.2-kb exogenous Ras mRNA transcript exhibited a 15- to 19-fold increase in $C57^{Ras}$ and MMEC/pH06T transfectants and a 33-fold increase in the tumor-derived MMEC/T_1Pr_1 cells, relative to that observed in the parental C57/MG and MMEC cells, respectively. Similarly, the expression of the 2.8-kb exogenous myc mRNA tran-

TABLE 1. Persistent Expression of Transfected Oncogenes in Mammary Epithelial Cell Lines

Cell Line	Transfection	Oncogene-specific mRNA Expression[a] (Arbitrary Scanning Units, ASU)[b]	
		1.2-kb Ras mRNA	2.8-kb myc mRNA
C57/MG	none	ND[c]	NT[c]
$C57^{Ras}$	v-Ras	15.6 ± 0.7	NT
MMEC	none	ND	ND
MMEC/pH06T	c-Ras	19.1 ± 2.8	NT
MMEC/myc_3	c-myc	NT	15.0 ± 2.6
MMEC/T_1Pr_1	Ras tumor	32.9 ± 3.1	NT
MMEC/myc_3-Pr_1	myc tumor	NT	41.7 ± 3.8

[a] Northern blot analysis using [^{32}P]-labeled nick-translated probes (2.9-kb Sac I fragment from c-Ha-Ras or 1.8-kb Sac I fragment from c-myc spanning the second exon).

[b] ASU per 20 μg RNA, mean ± SD, $n = 3$. RNA transcripts hybridizing to the probes represent exogenous transcripts due to expression of transfected oncogenes.

[c] ND: not detected; NT: not tested.

script was 15-fold higher in MMEC/myc_3 cells and 42-fold higher in MMEC/myc_3-Pr_1 cells, relative to that seen in the parental MMEC cells (TABLE 1).

Aberrant hyperplasia and tumorigenicity *in vivo*: The preneoplastic and neoplastic transformation was examined *in vivo* using the mammary fat pad transplantation technique[23–25] (TABLE 2). The transplantation of C57/MG and MMEC cells resulted in the formation of nonhyperplastic epithelial ducts at the transplantation site within 12–14 weeks after transplantation, and no tumors were observed even at 24–28 weeks after transplantation. MMEC/pH06T and MMEC/myc_3 cells produced an 80–90% incidence of hyperplasia at the 12–14-week time point and a comparable incidence of tumors at the 24–28-week time point. The transplantation of the tumor-derived MMEC/T_1Pr_1 and MMEC/myc_3-Pr_1 cells resulted in 100% tumor incidence within 8–10 weeks. Taken together, these observations extend and confirm our previous results, namely, that deregulated expression of

TABLE 2. Induction of Preneoplastic and Neoplastic Transformation in Oncogene-transfected Mammary Epithelial Cell Lines

Cell Line	Transfection	Incidence of Transformation[a]	
		Ductal Hyperplasia[b]	Adenocarcinoma[b]
C57/MG	none	0/10	0/10
$C57^{Ras}$	v-Ras	7/10	8/10
MMEC	none	0/10	0/10
MMEC/pH06T	c-Ras	9/10	8/10
MMEC/myc_3	c-myc	8/10	9/10
MMEC/T_1Pr_1	Ras tumor	0/10	10/10
MMEC/myc_3-Pr_1	myc tumor	0/10	10/10

[a] Examined 12–14 weeks after transplantation for the presence of ductal hyperplasia (preneoplastic transformation) or 24–28 weeks after transplantation for the presence of adenocarcinoma (neoplastic transformation).

[b] Number of fat pads with outgrowths/total number transplanted.

Ras and myc oncogenes confers tumorigenic transformation in mammary epithelial cells.[25,26] Furthermore, it is also evident that aberrant hyperplasia represents a cellular marker for preneoplastic transformation similar to that demonstrated for mammary epithelium transformed by chemical carcinogens or by the murine mammary tumor virus, the two prototypic initiators of rodent mammary tumorigenesis.[1,2,5,6,23,24,28,30,31]

Modulation of estrogen receptor status: The presence of estrogen receptor is critical for E_2-mediated proliferative signal transduction and resultant growth regulation.[3,4,7,16,17,21,22,35–37] The experiment designed to compare the amounts of estrogen receptor protein (ERP) in parental, oncogene-initiated, and oncogene-transformed mammary epithelial cells revealed a progressive decrease in ERP content corresponding with the expression of transformed cell phenotype. Thus, Ras- and myc-initiated cells exhibited about a 46% decrease, and the oncogene-transformed (i.e., tumor-derived) cells exhibited about a 75% decrease in ERP levels relative to that seen in the parental nontumorigenic cells (FIGURE 1).

Cellular metabolism of E_2: In the subsequent studies, experiments on oncogene-initiated mammary epithelial cells were designed to examine the extent of cellular metabolism of E_2 *in vitro*. These experiments have provided evidence that E_2 metabolism represents a specific and sensitive endocrine biomarker for preneoplastic transformation.

Our previous studies on noninvolved mammary tissue and on mammary epithelial cell lines have shown that initiation of the target cells by chemical carcinogens results in a specific increase in the C16α-hydroxylation pathway of E_2 metabolism.[24,29,31] Furthermore, 16α-OHE_1, the product of the C16α-hydroxylation pathway, is known to induce DNA damage and aberrant hyperproliferation in nontumorigenic C57/MG cells. In C57/MG cells initiated with the chemical carcinogen 7,12-dimethylbenz(*a*)anthracene (DMBA), 16α-OHE_1 is shown to enhance aberrant hyperproliferation. The 2-hydroxylated metabolite 2-OHE_1, in contrast to 16α-OHE_1, lacks genotoxicity and fails to induce aberrant hyperproliferation.[29,31] It was therefore important to examine whether deregulated expression of oncogenes alters cellular metabolism of E_2.

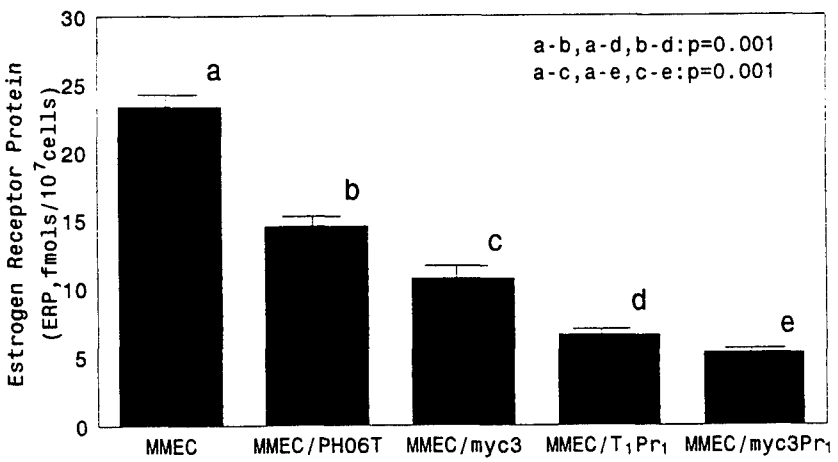

FIGURE 1. Estrogen receptor status in oncogene-transfected mammary epithelial cells.

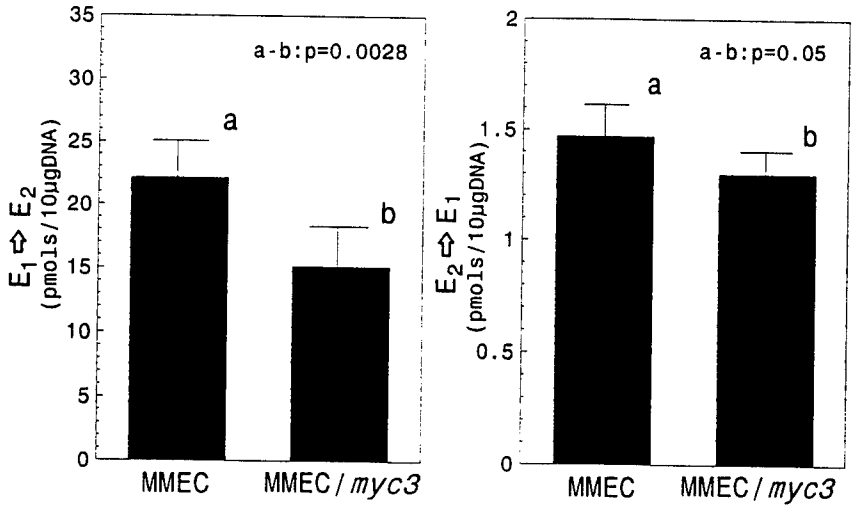

FIGURE 2. 17β-Hydroxysteroid dehydrogenase activity in *myc* oncogene–transfected mammary epithelial cells.

The experiment presented in FIGURE 2 was designed to examine the extent of 17β-hydroxysteroid dehydrogenase (17β-HSD) activity in the parental MMEC cells and the myc-initiated MMEC/myc_3 cells. The extent of E_1 to E_2 conversion (reductive activity of 17β-HSD) was suppressed by about 32% in MMEC/myc_3 cells relative to that observed in the parental MMEC cells. In contrast, the extent of E_2 to E_1 conversion (oxidative activity of 17β-HSD) was inhibited only by 12% in MMEC/myc_3 cells. These results suggest that myc oncogene–initiated cells are less dependent on E_2 than are the parental MMEC cells and, as a result, accumulate higher levels of E_1. Because E_1 functions as a common precursor for the formation of 2-OHE_1 or of 16α-OHE_1 via C2- and C16α-hydroxylation pathways, respectively, the oncogene-mediated alterations in E_2 metabolism were assessed by determining the C2/C16α-hydroxylation ratio (TABLE 3). Both oncogene-initiated

TABLE 3. Metabolism of 17β-Estradiol (E_2) in Oncogene-transfected Mammary Epithelial Cells

Cell Line	Transfection	E_2 Metabolism (C2/C16α-Hydroxylation)[a]	Relative Decrease (%)[b]
MMEC	none	3.05 ± 0.75[c]	—
MMEC/pH06T	c-Ras	0.23 ± 0.13[d]	92.4
MMEC/myc_3	c-myc	0.43 ± 0.03[e]	85.9
MMEC/T_1Pr_1	Ras tumor	0.14 ± 0.08[f]	95.4
MMEC/myc_3-Pr_1	myc tumor	0.26 ± 0.89[g]	91.5

[a] Determined by measuring ^3H_2O formation after a 48-h incubation with [C2-^{3}H]E_2 or with [C16α-^{3}H]E_2.

[b] Defined as [(transfectant − parental)/parental] × 100.

[c–g] Mean ± SD, n = 12. $c - d$, $c - f$: $P < 0.0001$; $d - f$: not significant; $c - e$, $c - g$: $P < 0.0001$; $e - g$: $P = 0.001$.

as well as oncogene-transformed cells exhibited about an 86–95% decrease in the C2/C16α-hydroxylation ratio relative to that seen in the parental MMEC cells. This alteration was in greater part due to a specific increase in the C16α-hydroxylation pathway, with a concomitant decrease in the C2-hydroxylation pathway (data not shown). These observations are consistent with those in our earlier studies on Ras oncogene- or carcinogen-initated cells.[26,29]

Aberrant hyperproliferation *in vitro*: Deregulated expression of Ras and myc oncogenes induces tumorigenic transformation in immortalized, but nontumorigenic mammary epithelial cells, as evidenced by rapidly growing tumors after *in vivo* transplantation of the stable transfectants.[25,26] Prior to the appearance of tumor, injected cells form hyperplastic outgrowths, similar to those induced by chemical carcinogens or by transforming retrovirus.[23–26,28] Thus, aberrant hyperplasia represents an early-occurring preneoplastic event in the multistage process of mammary carcinogenesis.[1–6]

The experiment shown in TABLE 4 was designed to identify a surrogate *in vitro* endpoint for aberrant hyperplasia that precedes tumorigenesis. The ability of cells to form tridimensional, anchorage-independent colonies represented this quantitative endpoint. The data clearly demonstrated that the parental nontumorigenic cells C57/MG and MMEC lacked anchorage-independent growth. In contrast, both oncogene-initiated (i.e., transfectants) and oncogene-transformed (i.e., tumor-derived) cells exhibited a specific and significant increase in anchorage-independent growth.

Modulation of preneoplastic transformation: Having demonstrated that oncogene-transfected cells exhibit altered E_2 metabolism and enhanced AIG *in vitro* prior to aberrant hyperplasia and tumorigenesis *in vivo*, it was now important to examine whether these perturbed biomarkers for preneoplastic transformation provide quantitative endpoints for the modulating effects of agents known to inhibit mammary tumorigenesis. Effective inhibition of perturbed biomarkers *in vitro* prior to tumorigenesis *in vivo* should provide evidence for the validity of the biomarkers as endpoints for primary prevention via inhibition of preneoplastic transformation. In the experiments utilizing Ras-initiated MMEC/pH06T and myc-initiated MMEC/myc$_3$ cells, the synthetic chemopreventive agents, TAM, 4-OH-TAM, and HPR, and the naturally occurring agents, I3C and EPA, were used as the test compounds. The data presented in FIGURES 3 and 4 indicate that all the test compounds effectively increased the C2/C16α-hydroxylation ratio of E_2 metabolism and decreased AIG. It is noteworthy that the synthetic antiestrogen

TABLE 4. Aberrant Hyperproliferation in Oncogene-transfected Mammary Epithelial Cells

Cell Line	Transfection	Anchorage-independent Growth (Number of Colonies)[a]	Relative Increase (× Control)[b]
C57/MG	none	0.5 ± 0.2	—
C57^{Ras}	v-Ras	38.6 ± 4.4	76.2
MMEC	none	0.8 ± 0.2	—
MMEC/pH06T	c-Ras	27.2 ± 4.0	33.0
MMEC/myc$_3$	c-myc	28.0 ± 2.0	34.0
MMEC/T$_1$Pr$_1$	Ras tumor	125.0 ± 2.7	155.2
MMEC/myc$_3$Pr$_1$	myc tumor	132.0 ± 5.4	164.0

[a] Number of anchorage-independent colonies per 1.0×10^4 cells; mean ± SD, $n = 18$.
[b] Defined as (transfectant − parental)/parental.

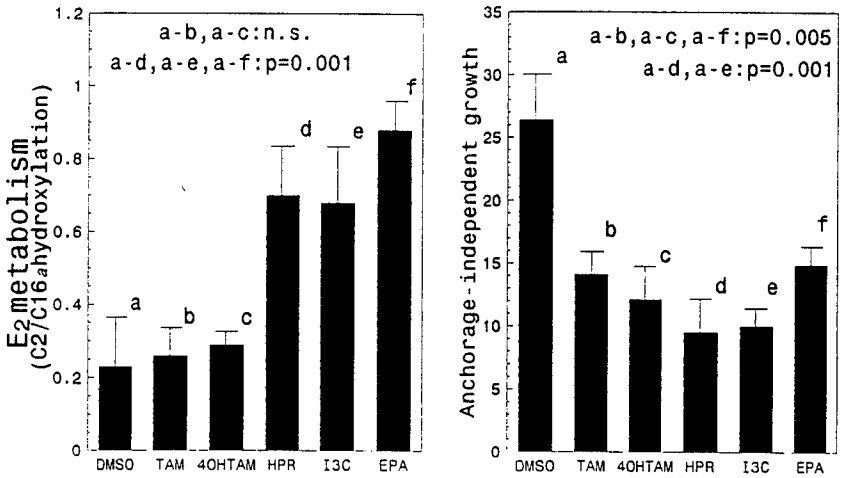

FIGURE 3. Effect of chemopreventive agents on 17β-estradiol metabolism and anchorage-independent growth in *Ras* oncogene–transfected mammary epithelial cells.

TAM and its major metabolite 4-OH-TAM were substantially less effective in altering E_2 metabolism than were HPR, I3C, and EPA. It is therefore conceivable that the effects of these agents on AIG may be manifested via distinct mechanisms. In this context, it is noteworthy that all the test compounds used in these experiments are documented to inhibit the development of either mammary tumor virus–initiated or chemical carcinogen–initiated rodent mammary tumors *in vivo*.[6,38–43] Thus, effective downregulation of biomarkers for preneoplastic transformation *in*

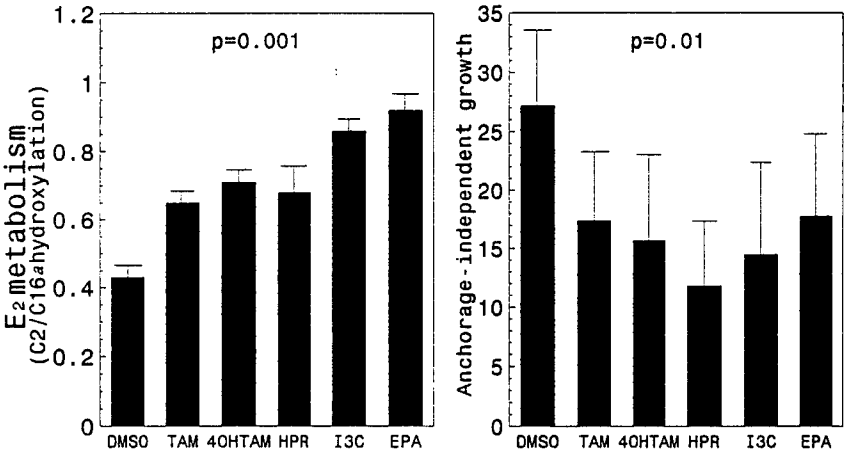

FIGURE 4. Effect of chemopreventive agents on 17β-estradiol metabolism and anchorage-independent growth in *myc* oncogene–transfected mammary epithelial cells.

vitro provides evidence to support the validity of the biomarkers as endpoints for chemopreventive intervention.

CONCLUSIONS

The data presented in this overview permit the following conclusions:

(1) Deregulated expression of Ras and myc oncogenes confers preneoplastic and neoplastic transformation in mammary epithelial cells as evidenced by a high incidence of aberrant hyperplasia and tumorigenicity *in vivo*.
(2) The stable transfectants exhibit persistent expression of oncogene-specific mRNA transcripts, alteration in E_2 metabolism, and enhancement of anchorage-independent growth *in vitro*.
(3) The mammary epithelial cells exhibit a progressive decrease in estrogen receptor status corresponding with tumorigenic transformation.
(4) Altered E_2 metabolism and enhanced anchorage-independent growth provide sensitive and specific biomarkers for induction and modulation of oncogene-induced mammary preneoplastic transformation.

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Transcription of Cyclooxygenase-2 Is Enhanced in Transformed Mammary Epithelial Cells[1]

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ABSTRACT

Cancers form more prostaglandins than the normal tissues from which they arise. Cyclooxygenase-2 (prostaglandin H synthase-2, PGHS-2, EC 1.14.99.1), an enzyme that catalyzes the formation of prostaglandins from arachidonic acid, is inducible in epithelial cells. We investigated whether transformation of mammary cells was associated with up-regulation of Cox-2 as a basis for increased production of prostaglandin E_2 (PGE_2) by these cells. This hypothesis was tested in two pairs of mammary cell lines between which the mode of transformation (viral *versus* oncogene) differed. Virally transformed RIII/Pr1 cells, which are highly tumorigenic in mice, produced markedly increased amounts of PGE_2 compared to virally initiated RIII/MG cells, a weakly tumorigenic strain. Cox-2 mRNA and protein were increased concomitantly in RIII/Pr1 cells. Similarly, Ras-induced transformation of C57/MG cells resulted in increased levels of Cox-2 mRNA and protein and increased production of PGE_2. Nuclear run-offs revealed increased rates of Cox-2 transcription in the virally transformed and oncogene-transformed cell lines. Transient transfection experiments demonstrated that the oncogenes *src* and *ras* up-regulated Cox-2 promoter activity. Src-mediated up-regulation of Cox-2 promoter activity was suppressed by dominant negative ras. Our data indicate that cellular transformation is associated with enhanced transcription of Cox-2 and increased production of PGE_2.

INTRODUCTION

It has been known for many years that cancers form more prostaglandins than the normal tissues from which they arise. Examples of tumors that produce increased levels of prostaglandins include cancers of the breast, head and neck, and colon (1–6). Prostaglandins such as PGE_2[3] affect cell proliferation and alter the response of the immune system to malignant cells so that overproduction of prostaglandins could favor malignant growth (7). Moreover, inhibitors of prostaglandin synthesis (*e.g.*, aspirin, sulindac, and curcumin) protect against carcinogenesis (7–11). One strategy for modulating carcinogenesis might be to prevent the up-regulation of prostaglandin synthesis in premalignant and malignant tissue. It is important, therefore, to elucidate the underlying mechanism that accounts for the increased production of prostaglandins in cancer.

Cyclooxygenase catalyzes the formation of prostaglandins from arachidonic acid. Results from recent studies have established the presence of two distinct Cox enzymes, a constitutive enzyme (Cox-1) and an inducible isoform (Cox-2; Refs. 12 and 13), the products of separate but related genes. The *Cox-2* gene has been characterized as an early response gene that, like c-*fos* and c-*jun*, is induced rapidly following stimulation of quiescent cells. The biosynthesis of Cox-2 protein is stimulated by serum, growth factors, cytokines, and phorbol esters (14–16), whereas the constitutive isoform Cox-1 is unaffected by these factors. Increased expression of Cox-2 is associated with increased production of prostaglandins, including PGE_2.

It is reasonable to postulate that increased expression of Cox-2 plays a major role in the overproduction of prostaglandins in malignant tissue. Hong *et al.* (17) showed nearly 20 years ago that Cox activity was increased in transformed cells. Moreover, increased levels of Cox-2 are detected in several cell types, including epithelial cells in colon tumors (18). Since we now know that Cox-2 is readily inducible whereas Cox-1 is constitutively expressed, it seemed likely that the increase in Cox activity in transformed cells reflected up-regulation of Cox-2. In the present work, we investigated the effects of transformation on expression of Cox-2 and biosynthesis of prostaglandins in mammary epithelial cells. Our data show that cellular transformation is associated with enhanced transcription of Cox-2 and increased production of PGE_2.

MATERIALS AND METHODS

Materials. DMEM/F-12 and FBS were from Life Technologies, Inc. (Grand Island, NY). Enzyme immunoassay reagents for PGE_2 assays were from Cayman Co. (Ann Arbor, MI). Random-priming kits were from Boehringer Mannheim Biochemicals (Indianapolis, IN). Nitrocellulose membranes were from Schleicher & Schuell (Keene, NH).

Cell Lines. The C57/MG and RIII/MG cell lines were established from noncancerous mammary tissue from C57BL/6J and RIII mouse strains, respectively, according to published procedures (19, 20). The C57Ras cell line is a stable transfectant of C57/MG that expresses the V-Ha-*Ras* oncogene. The transfection was performed according to the published procedure using electroporation (20, 21). The expression plasmid contained V-Ha-Ras under the transcriptional control of MLV-LTR and the selectable marker Neo^r, which confers resistance to the antibiotic geneticin (G418). The RIII/Pr1 cell line was established from a primary tumor obtained from a multiparous female RIII mouse. Routinely, the four cell lines were maintained in DMEM/F-12 containing 4 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 100 μg/ml gentamicin, and 4 μg/ml amphotericin B. This basal medium was supplemented with 10% heat-inactivated FBS and 5 μg/ml insulin. The stock cell lines were routinely subcultured at a 1:10 dilution when they reached approximately 70% confluence. C57Ras cells were maintained in the presence of 400 μg/ml G418.

PDT. The PDTs for the four cell lines were determined from the linear portions of the growth curves generated for at least 5 days after seeding 5.0×10^3 cells/25 cm^2 (20, 21).

AIG. The ability of the four cell lines to undergo AIG was evaluated by determining the number of anchorage-independent, 3-dimensional colonies formed in 0.33% agar 14 days after seeding at an initial density of 1.0×10^3 cells/well (20, 21).

Tumorigenicity. The tumorigenic potential of the four cell lines was evaluated by determining the number of palpable tumors that arose after injection of 1.0×10^5 cells as a single 20-μl bolus into parenchyma-free mammary fat pads of syngeneic recipients (22, 23). Tumor incidence was determined 12 weeks after injection of the cells.

PGE_2 Production. Cells were plated in six-well plates as described above and grown for 3 days. Spent medium was then replaced with fresh medium.

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[3] The abbreviations used are: PGE_2, prostaglandin E_2; Cox-2, cyclooxygenase-2; PDT, population doubling time; AIG, anchorage-independent growth; FBS, fetal bovine serum; SSPE, sodium chloride-sodium phosphate-EDTA buffer; NSAID, nonsteroidal anti-inflammatory drug; CRE, cyclic AMP response element.

Twenty-four h later, the culture medium was collected to determine amounts of PGE_2 secreted by these cells. Medium was obtained from each well and centrifuged to sediment cell debris; the supernatants were added to fresh tubes and frozen at −80°C until assay. Supernatants were assayed by enzyme immunoassay for PGE_2 spontaneously released from the cells (24).

Western Blotting. Lysates were prepared by treating cells with lysis buffer consisting of 150 mM NaCl, 100 mM Tris-buffered saline, 1% Tween 20, 50 mM diethyldithiocarbamate, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride. Lysates were sonicated twice for 20 s on ice and centrifuged at 10,000 × *g* for 10 min to sediment the particulate material. Protein concentration of the supernatant fractions was measured by the method of Lowry *et al.* (25), and the supernatants were stored at −80°C until used. SDS-PAGE was performed under reducing conditions on 10% polyacrylamide gels as described by Laemmli (26). The resolved proteins were transferred onto nitrocellulose sheets as detailed by Towbin *et al.* (27). The nitrocellulose membrane was then incubated with a rabbit polyclonal antibody raised against the unique 18-amino acid sequence from the carboxyl-terminal portion of human Cox-2 that does not react with Cox-1 (28). Nitrocellulose membranes were also probed with a polyclonal anti-Cox-1 antibody (Oxford Biomedical Research, Oxford, MI). The membrane was subsequently incubated with a goat antirabbit antibody conjugated to alkaline phosphatase and developed as described previously (29). A computer densitometer (Molecular Dynamics, Sunnyvale, CA) was used to determine the density of the bands.

Northern Blotting. To prepare total cellular RNA, cell monolayers were washed and then directly lysed in 4 mol/liter guanidinium isothiocyanate solution. RNA was then isolated by phenol-chloroform extraction according to Chomczynski and Sacchi (30). For Northern blots, 6 μg of total cellular RNA per lane were electrophoresed in formaldehyde-containing 1.2% agarose gels and transferred to nylon-supported membranes. After baking, membranes were prehybridized for 3 h and then hybridized in a solution containing 50% formamide, 5× SSPE, 5× Denhardt's solution, 0.1% SDS, and 100 μg/ml single-stranded salmon sperm DNA. Hybridization was carried out for 16 h at 42°C with a radiolabeled murine Cox-2 cDNA probe (TIS 10) that was kindly provided by Dr. Harvey Herschman (University of California, Los Angeles). After hybridization, membranes were washed for 20 min at room temperature in 2× SSPE-0.1% SDS, twice for 20 min in the same solution at 55°C, and twice for 20 min in 0.1× SSPE-0.1% SDS at 55°C. Washed membranes were then subjected to autoradiography. To verify equivalency of RNA loading in the different lanes, the blot was stripped of radioactivity and rehybridized to determine levels of 18S rRNA. Cox-2 and 18S rRNA probes were labeled with [^{32}P]dCTP by random priming. The signal level of the bands was quantified by densitometry.

Nuclear Run-off. For each of the four cell lines, 2.5 × 10^5 cells were plated in four T150 dishes. Cells were grown as described above until they were approximately 70% confluent. Nuclei were isolated and stored in liquid nitrogen. For the transcription assay, nuclei (1.0 × 10^7) were thawed and incubated in reaction buffer containing 100 μCi of uridine 5′[α^{32}P]triphosphate and unlabeled nucleotides. After 30 min, labeled nascent RNA transcripts were isolated. The TIS10 and 18S rRNA cDNAs were immobilized onto nitrocellulose and prehybridized overnight in hybridization buffer. Hybridization was carried out at 42°C for 24 h using at least 5 × 10^5 cpm of labeled nascent RNA transcripts. The filters were washed twice with 2× SSC buffer for 1 h at 55°C and then treated with 10 mg/ml RNase A in 2× SSC at 37°C for 30 min, dried, and autoradiographed.

Plasmids. The Cox-2 promoter constructs TIS10L, TIS10-80, and TIS10-40 were generously provided by Dr. Harvey Herschman (University of California, Los Angeles). These constructs contain 963, 80, and 40 bases 5′ of the transcription start site of Cox-2, respectively. The v-src expression vector was a gift from Dr. David Foster (Hunter College). The plasmid expressing wild-type CREB was from Dr. James Leonard (Strang Cancer Prevention Center). The Ras constructs were gifts from Dr. Geoffrey Cooper (Harvard University). The c-Jun expression vector was provided by Dr. Tom Curran (Roche Laboratories).

Transient Transfection and Reporter Assays. C57/MG cells were seeded at a density of 4 × 10^4 cells/well in six-well dishes and grown to 30–40% confluence in DMEM/F-12 containing 10% FBS. Two μg of plasmid DNA were introduced into cells using 8 μg of LipofectAMINE as per the manufacturer's instructions. After 5 h of incubation, the medium was replaced with DMEM/F-12 under serum-free conditions. Luciferase activity was measured in cellular extract 24 h later.

Luciferase activity was measured as follows. Each well was washed twice with PBS. One hundred μl of 1× lysis buffer (Analytical Luminescence Laboratories, San Diego, CA) were added to each well for 15 min. The lysate was centrifuged for 1 min at 4°C. The supernatant was used to assay luciferase activity with a Monolight 2010 luminometer (Analytical Luminescence Laboratories, San Diego, CA) according to the manufacturer's instructions. Luciferase activities were normalized to protein concentrations.

Statistics. Comparisons between groups were made by the Student's *t* test. A difference between groups of $P < 0.05$ was considered significant.

RESULTS

Characterization of Cell Lines. The ability of murine mammary tumor virus and V-Ha-Ras to initiate transformation of mammary epithelial cells was evaluated by determining aberrant proliferation *in vitro* by assays of PDT and AIG and *in vivo* by tumorigenicity. As shown in Table 1, RIII/Pr1 and C57Ras cells exhibited about 12 and 29% shorter PDTs than RIII/MG and C57/MG cells, respectively. Increased AIG also was observed in the RIII/Pr1 and C57Ras cell lines compared to the RIII/MG and C57/MG cell lines. To correlate these measurements of aberrant proliferation *in vitro* with tumor formation *in vivo*, we assessed the tumorigenic potential of the four different cell lines. Tumors were detected in 90% of animals injected with RIII/Pr1 cells compared with 30% treated with RIII/MG cells. Eighty % of mice treated with C57Ras cells developed tumors. In contrast, the C57/MG cells did not form tumors. These differences in tumorigenicity between transformed and nontransformed lines are consistent with the observed differences in PDT and AIG.

Transformation Is Associated with Increased Production of PGE_2. We investigated the possibility that cellular transformation would affect the production of PGE_2. As shown in Fig. 1, spontaneous production of PGE_2 was increased by nearly 12-fold in virally transformed RIII/Pr1 cells compared to virally initiated RIII/MG cells. A similar comparison was made in the C57/MG and C57Ras cell lines. Production of PGE_2 was about 2.5-fold greater in the C57Ras compared to the C57/MG cell line.

Cox-2 Is Up-Regulated in Transformed Mammary Epithelial Cells. To determine whether the above differences in production of PGE_2 could be related to differences in levels of cyclooxygenase, Western blotting of cell lysate protein was carried out. Fig. 2 shows immunoblots for cell lysates prepared from RIII/MG, RIII/Pr1, C57/MG, and C57Ras cells. Cox-2 exists as a doublet due to differences in glycosylation (31). Greater amounts of Cox-2 were detected in the two highly tumorigenic cell lines (RIII/Pr1 and C57Ras) than in their respective partner cell lines (RIII/MG and C57/MG). These differences in cellular expression of Cox-2 were reflected by the differences in spontaneous PGE_2 production shown in Fig. 1. Cox-1 was not detectable by immunoblotting in any of the cell lines under study (data not shown).

Table 1 *Biological characteristics of mammary cell lines*

Cell Line	Biomarkers of Transformation		
	PDT[a] (h)	AIG[b]	T[c]
RIII/MG	30.5 ± 1.1[d]	28.9 ± 5.7	6/20 (30%)
RIII/Pr1	26.8 ± 1.8[e]	92.0 ± 8.9	18/20 (90%)
C57/MG	37.4 ± 2.3[f]	0.5 ± 0.1	0/20 (0%)
C57Ras	26.4 ± 1.9[g]	35.1 ± 2.5	8/10 (80%)

[a] Determined from cell number in log phase cultures. Values represent mean ± SD; $n = 4$.
[b] Number of "soft" agar colonies. Values represent mean ± SD; $n = 6$.
[c] Adenocarcinomas 12 weeks after mammary fat pad transplantation.
[d-e] $P = 0.04$.
[f-g] $P = 0.01$.

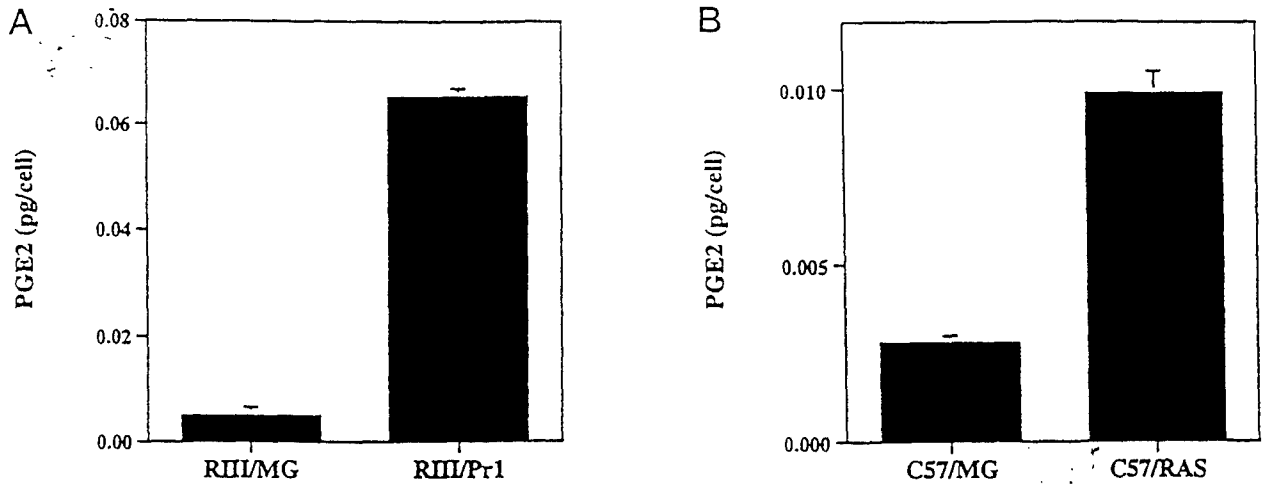

Fig. 1. PGE_2 production is increased in transformed mammary epithelial cells. Postculture medium was collected after 24 h, as described in "Materials and Methods" and assayed for PGE_2 by enzyme immunoassay. *A*, virally transformed RIII/Pr1 cells produced higher levels of PGE_2 than virally initiated RIII/MG cells. *B*, Ras-induced transformation of C57/MG cells resulted in increased synthesis of PGE_2 compared to synthesis in nontransformed cells. *Columns*, mean; *bars*, SD; $n = 6$.

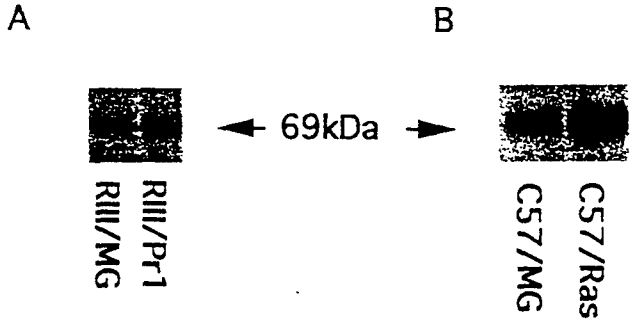

Fig. 2. Transformation is associated with increased amounts of Cox-2. Total cell lysates were prepared from RIII/MG and RIII/Pr1 cells (*A*) and from C57/MG and C57Ras cells (*B*) under conditions stated in the legend to Fig. 1. Lysate protein (25 μg/lane) was loaded onto a 10% SDS-polyacrylamide gel, electrophoresed, and subsequently transferred onto nitrocellulose. Incubation with an antibody specific for Cox-2 revealed higher levels of Cox-2 in RIII/Pr1 than in RIII/MG cells; higher levels of Cox-2 were detected in C57Ras than in C57/MG cells. The doublet corresponding to Cox-2 was quantified by densitometry. *A*, RIII/MG, 58 arbitrary units; RIII/Pr1, 143 arbitrary units. *B*, C57/MG, 340 arbitrary units; C57Ras, 693 arbitrary units.

Increased amounts of Cox-2 enzyme in the two highly tumorigenic cell lines could reflect either increased protein synthesis or decreased degradation. To examine activation of the *Cox-2* gene as a possible mechanism, we determined steady-state levels of Cox-2 mRNA by Northern blotting. As shown in Fig. 3, higher levels of Cox-2 mRNA were detected in the RIII/Pr1 than in the RIII/MG cells. Similarly, higher levels of Cox-2 mRNA were found in the C57Ras than in the C57/MG cells.

Transcription of Cox-2 Is Enhanced in Transformed Mammary Epithelial Cells. Differences in levels of mRNA could reflect altered rates of transcription or mRNA stability. To distinguish between these two possibilities, nuclear run-offs were performed. As shown in Fig. 4, we observed higher rates of synthesis of nascent Cox-2 mRNA in the two transformed cell lines consistent with the differences observed by Northern blotting.

To further investigate the importance of oncogenes in modulating the expression of Cox-2, transient transfections were performed using src and ras expression vectors cotransfected with Cox-2 luciferase reporter constructs. As shown in Fig. 5, the *src* and *ras* oncogenes up-regulated Cox-2 promoter activity. Induction of Cox-2 promoter activity was localized to a region between -80 and -40 nucleotides 5′ of the Cox-2 transcription start site. Furthermore, src-mediated stimulation of Cox-2 promoter activity was suppressed by dominant negative ras (Fig. 5*C*).

The regulation of Cox-2 expression in mammary epithelial cells was investigated further by determining the effects of the transcription factors, CREB and c-Jun, on Cox-2 promoter activity. The basal promoter activity of TIS10L was higher than TIS10-80 or TIS10-40.

Fig. 3. Levels of Cox-2 mRNA are increased in transformed mammary epithelial cells. Total cellular RNA was isolated from RIII/MG and RIII/Pr1 cells (*A*) and from C57/MG and C57Ras cells (*B*), 24 h after changing the medium as described in the legend to Fig. 1. Each lane contained 6 μg of RNA. The Northern blots were probed sequentially with probes that recognized Cox-2 mRNA (4.3-kb band) and 18S rRNA. Higher levels of Cox-2 mRNA were detected in the transformed mammary epithelial cells.

Fig. 4. Transformation of mammary epithelial cells is associated with increased rates of Cox-2 transcription. Nuclei were isolated from the four different cell lines following treatment with DMEM/F-12 containing 10% FBS for 24 h. Nuclear run-offs were performed as described in "Materials and Methods." The Cox-2 and 18S rRNA cDNAs were immobilized onto nitrocellulose membranes and hybridized with labeled nascent RNA transcripts from the different cell lines. *A*, RIII/MG and RIII/Pr1 blots; *B*, C57/MG and C57/Ras blots.

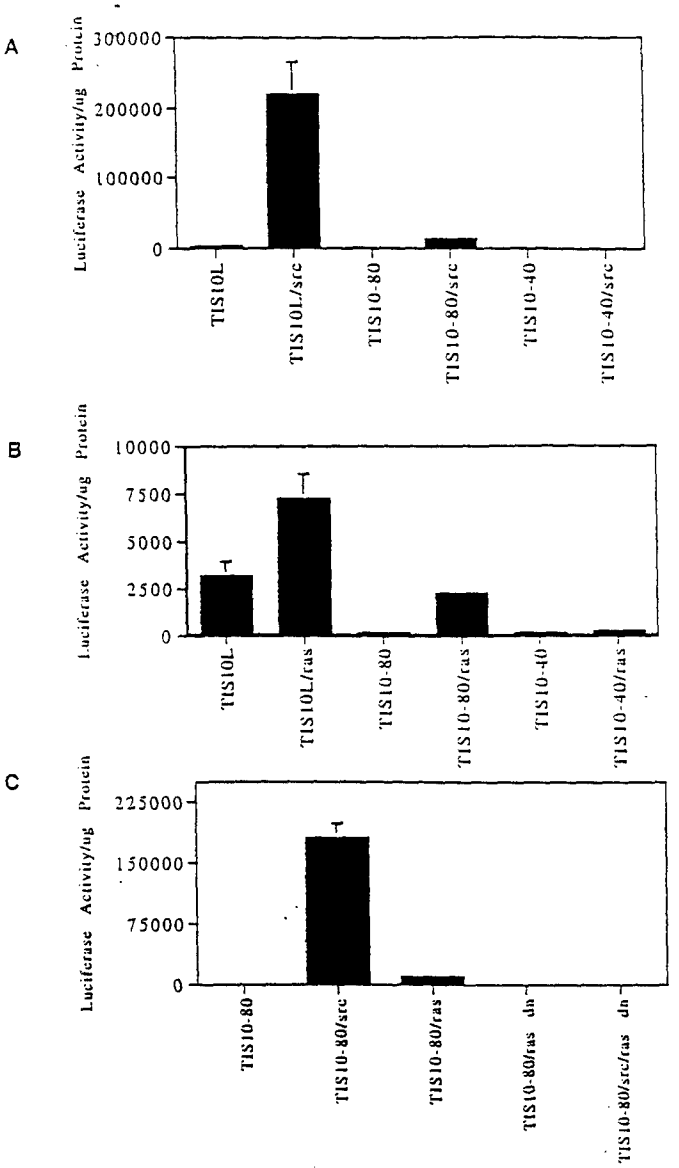

Fig. 5. Oncogene-mediated induction of Cox-2 promoter activity. The C57/MG cell line was transfected with a series of Cox-2 luciferase deletion constructs: TIS10L (−963), TIS10-80 (−80), and TIS10-40 (−40). One μg of the TIS10 reporter plasmid was used in all experiments. *A*, cells were cotransfected with v-src (1 μg); *B*, cells were cotransfected with ras expression vector (1 μg); *C*, cells were cotransfected with src (0.5 μg), ras (0.5 μg), dominant negative (*dn*) ras (0.5 μg) or src (0.5 μg), and dominant negative ras (0.5 μg). The total amount of DNA in each reaction was kept constant by using corresponding empty expression vectors. Six wells were used for each transfection condition. *Columns*, mean; *bars*, SD.

Both CREB and c-Jun enhanced Cox-2 promoter activity, but the extent and locus of induction differed for these two transcription factors. c-Jun was a more potent inducer of Cox-2 promoter activity than CREB (Fig. 6). For example, c-Jun caused about 10-fold greater activation of TIS10L than CREB (57.8- *versus* 5.1-fold increase). Also, whereas CREB-mediated induction of Cox-2 was localized to a region between −80 and −40 nucleotides 5′ of the Cox-2 transcriptional start site, c-Jun activation of Cox-2 promoter activity was observed in both −80 and −40 Cox-2 luciferase deletion constructs (Fig. 6).

DISCUSSION

In this study, we showed that virus and oncogene-mediated transformation of mammary epithelial cells is associated with increased expression of the *Cox-2* gene, resulting in enhanced production of prostaglandins. This observation is important for a variety of reasons. First, our finding that *Cox-2* gene expression is enhanced in transformed cells provides a mechanism for the phenomenon of increased production of prostaglandins by transformed epithelial cells. Although transformed cells have been shown to overproduce prostaglandins, the underlying mechanism(s) responsible for this effect had not been elucidated (17, 32, 33). It is important to point out, however, that cellular transformation may result in other changes that could contribute to increased production of prostaglandins. For example, altered phospholipase A_2 activity might also be important for explaining differences in prostaglandin synthesis (34).

There is increasing evidence that Cox-2 is up-regulated in human tumors compared with adjacent normal tissue (18, 35, 36). Increased levels of Cox-2 in tumors reflect enzyme induction in both epithelial and non-epithelial cells (*e.g.*, inflammatory cells; Ref. 18). Up-regulation of Cox-2 in epithelial cells in tumors could be a direct result of cellular transformation or a consequence of stimulation by cytokines or growth factors. Our cell culture data provide evidence that up-regulation of Cox-2 is a direct consequence of cellular transformation of epithelial cells. This result does not exclude the possibility that exogenous stimuli, such as cytokines and growth factors, contribute to the increased levels of Cox-2 in neoplastic tissue.

Cox-catalyzed reactions may be important for carcinogenesis via several different mechanisms. For example, although the cytochrome P-450 family of enzymes is recognized widely for catalyzing oxidative reactions that convert xenobiotics to reactive electrophiles, Cox also converts a broad array of chemicals to mutagens (37). In addition to catalyzing oxidation reactions to produce mutagens, Cox-catalyzed reactions may predispose to carcinogenesis via other mechanisms. Thus, the prostaglandins synthesized by Cox impair immune surveillance and killing of malignant cells (38), and overexpression of Cox-2 in epithelial cells inhibits apoptosis (39). Prolonged survival of abnormal cells favors the accumulation of sequential genetic changes, which could result in tumor progression. Compounds that inhibit Cox, therefore, may decrease the formation of mutagens and at the same

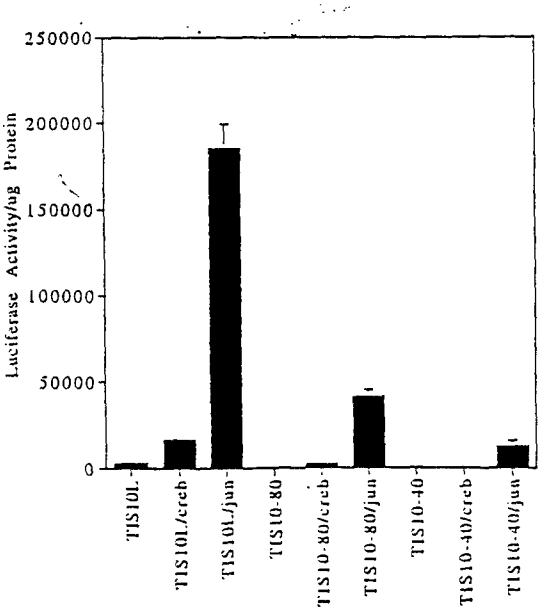

Fig. 6. Overexpression of c-Jun and CREB enhances Cox-2 promoter activity. C57/MG cells were transfected with 1.0 μg of Cox-2 luciferase deletion constructs TIS10L (−963), TIS10-80 (−80), and TIS10-40 (−40), along with the expression vectors for c-Jun (1.0 μg) or CREB (1.0 μg). The total amount of DNA in each reaction was kept constant by using corresponding empty expression vectors. Six wells were used for each transfection condition. *Columns*, mean; *bars*, SD.

time enhance processes such as immune surveillance and apoptosis, which tend to destroy initiated cells. Indeed, NSAIDs (8), retinoids (40), and curcumin (41) all inhibit both Cox-mediated metabolism of arachidonic acid and carcinogenesis.

Cox has both cyclooxygenase and peroxidase activities. Aside from being important for prostaglandin synthesis, the peroxidase function contributes to the activation of procarcinogens. Drugs such as NSAIDs inhibit the cyclooxygenase but not the peroxidase function of Cox, which potentially limits their effectiveness. Our observation that transcription of Cox-2 is enhanced in transformed cells is likely to be important for understanding why inhibitors of Cox, such as NSAIDs, do not work more effectively in preventing or treating cancer. Increased transcription of Cox-2 could result in the synthesis of functional enzyme despite treatment with drugs aimed at inhibiting enzyme function. Thus, chemicals that interfere with the signaling mechanism(s) responsible for up-regulation of Cox-2 should decrease levels of Cox-2 and thereby inhibit carcinogenesis at least as effectively as drugs that inhibit the cyclooxygenase activity of Cox. A detailed understanding of the regulation of Cox-2 transcription should provide important insight into possible approaches to block the expression of Cox-2 in transformed cells and tumors, thereby inhibiting all functions of this enzyme.

In an effort to elucidate the transcriptional regulation of Cox-2 in epithelial cells, we identified a region between −80 and −40 nucleotides 5′ of the Cox-2 transcription start site, which mediates induction of Cox-2 by v-src and ras. These results are consistent with the findings of Xie *et al.* (42–44), which showed that v-src induced expression of the *Cox-2* gene via a consensus CRE in this region. Other enhancer elements also must be present because we made the novel observation that c-Jun activated Cox-2 promoter activity in both −80 and −40 Cox-2 luciferase deletion constructs. The finding that src-mediated stimulation of Cox-2 promoter activity was suppressed by dominant negative ras provides insight into potential approaches to down-regulating Cox-2 expression in transformed cells. Inhibitors of farnesylation (45) or tyrosine kinase (46) may suppress the expression of Cox-2. It is important to point out that AP-1 transcription factors also can modulate transcription via a CRE (47). Further studies are needed to determine whether AP-1 factors contribute to the up-regulation of Cox-2 in transformed epithelial cells. Dexamethasone, a compound that suppresses AP-1-mediated gene expression, down-regulates levels of Cox-2, but it is unclear whether this occurs via a CRE-dependent mechanism. It is of considerable interest that retinoids and curcumin, known chemopreventive agents, inhibit the metabolism of arachidonic acid (40, 41) and antagonize AP-1-mediated transcription (48, 49). Whether these compounds down-regulate levels of Cox-2 requires further investigation.

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NEGATIVE GROWTH REGULATION OF HER-2/NEU ONCOGENE-TRANSFORMED HUMAN MAMMARY EPITHELIAL CELLS BY A GREEN TEA POLYPHENOL.

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Up-regulation of HER-2/neu oncogene expression is associated with neoplastic transformation of mammary epithelial cells. Although naturally-occurring phytochemicals inhibit rodent organ site cancer, clinical relevance of animal data remains equivocal. Present experiments were designed on a human *in vitro* model for mammary carcinogenesis to examine the preventive efficacy and to identify possible mechanisms of action of the green tea polyphenol (-)epigallocatechin gallate (EGCG). The preneoplastic HER-2/neu expressing human mammary epithelial 184-B5/HER cells represented the experimental system, while altered cell cycle progression, extent of cellular apoptosis and modulation in select cell cycle regulatory genes represented the quantitative parameters. A seven day exposure of 184-B5/HER cells to EGCG produced a progressive anchorage-dependent growth inhibition ($IC_{50} \approx 3.8 \pm 0.4 \mu M$). A twenty four hour exposure to EGCG resulted in cell cycle arrest in the S and G_2+M phases and induction of the Sub G_0 (apoptotic) peak. This treatment also induced a 36.6% (P=0.002) and a 65.7% (P=0.009) inhibition in constitutive immunoreactivity to Bcl-2 and to phosphorylated tyrosine respectively. The antiproliferative activity of EGCG in the 184-B5/HER cells may in part be due to inhibition of HER-2/neu-mediated signal transduction and induction of Bcl-2-dependent cellular apoptosis. [Support: Grant #DAMD17-94-J-4208, CA 29502, Indo-US Fulbright Fellowship #17267 and philanthropic support to the Strang Cancer Prevention Center]

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SESSION 1A

Oncogenes and cancer

Chairperson : S.E. Hasnain

Role of cell cycle regulation and apoptosis in prevention of human breast cancer by phytochemicals

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Overexpression of HER-2/neu proto-oncogene confers tumorigenic transformation in the mammary tissue of transgenic mice and in immortalized human mammary epithelial cells. This proto-oncogene also represents a prognostic marker for early relapse of human breast cancer. Tumorigenic transformation associated aberrant hyperproliferation and cellular apoptosis represent two events in multistep carcinogenesis whose modulation may provide quantitative biomarkers for effective prevention. Dysregulation of either apoptosis or anti-apoptotic genes have been shown to play a significant role in multistage carcinogenesis. The naturally-occurring phytochemicals are known to lower the risk of organ site cancer in rodent models. The present study was undertaken to identify phytochemical-mediated modulation in cell cycle regulatory gene expression and the role of cellular apoptosis in prevention of human breast cancer.

The experiments were designed to:1) determine the growth kinetics of immortalized non-tumorigenic human mammary cells 184-B5 and HER-2/neu proto-oncogene initiated 184-B5/HER cells, 2) demonstrate the response of these two cell lines to selected naturally-occurring phytochemicals and 3) identify modulation of cell cyclye regulatory gene expression and role of cellular apoptosis in downregulation of aberrant hyperproliferation. Fluorescence assisted cell sorting (FACScan) and immunocytometry was utilized to look at the markers of cell cycle progression (Bcl-2, p53, PCNA, P16,) with particular attention to apoptosis.

The parental as well as proto-oncogene initiated human mammary epithelial cells exhibited progressive time dependent increase in growth. The 184-B5 cells exhibited a population doubling time (PDT) of 32.8± 1.6 hr and about 24 fold increase relative to initial seeding density at 8-day post seeding. In contrast, 184-B5/HER cells showed a 34% decrease in PDT (0.001) and a 38 fold increase in number at 8-day post seeding. These results suggest that overexpression of HER-2/neu oncogene induces aberrant hyperproliferation in non-tumorigenic human mammary epithelial cells 184-B5.

A continous 7-day treament with indole-3-carbinol (I3C, cruciferous glucosinolate), (-) epigallocatechin gallate (EGCG, a green and black tea polyphenol) and genestein (GEN, a soy isoflavone) showed differential growth arrest to the two cell lines. Effective inhibitory concentrations of the phytochemicals were at least 3-

10 fold higher for 184-B5/HER relative to 184-B5. Treatment of 184-B5/HER cells for 24 hr with non-toxic doses of I3C, EGCG or GEN resulted in induction of apoptosis and variable growth arrest of cells in S and/or, G2/M phases of cell, cycle tabulated below:

Table 1. Cell cycle distribution of 184-B5/HER cells treated with phytochemicals

		% Distribution of Cells[b]		
Agent[a]	APO	$G_0 + G_1$	S	$G_2 + M$
DMSO (0.1%)	5.1±1.3	73.8±8.8	15.4±4.5	10.8±4.6
13C (200μM)	13.1±2.8	62.7±22.1	19.1±8.6	18.2±14.7
EGCG (22μM)	16.4±1.6	47.0±6.6	27.0±5.3	26.0±1.4
GEN (7.5μM)	24.9±1.8	39.0±18.2	53.8±15.2	7.1±3.1

[a]Cells treated with the agent for 24 hours [b]Values are mean ±SD, n=8

The down regulation of growth observed by the three phytochemicals correlates with decrease in Bcl-2 and PCNA protiens concomitant with induction of apoptosis shown in table below.

Table 2. Effect of phytochemicals on cell cycle regulatory gene expression in 184-B5/HER cells

Regulatory gene product	Fluorescence intensity[a] (arbitrary fluorescence unit[b], overall Log)			
	DMSO	13C	EGCG	GEN
Bcl-2	69.2±3.6	60.9±1.3	43.9±1.4	43.4±1.4
p53	13.8±1.9	12.7±1.9	12.3±1.4	14.2±2.0
PCNA	11.3±0.5	8.8±0.7	8.9±0.2	8.0±0.1
P16	14.1±0.6	10.3±0.2	9.5±0.9	9.2±0.4

[a]determined from FITC labelled monoclonal antibodies, [b]values are mean± SD, n=8/treatment group.

Our results indicate that impaired cellular homeostasis induced by HER-2/neu can be modulated by the phytochemicals via regulation of cell cycle progression. Aberrant hyperproliferation, modulation of cell cycle regulatory gene expression and celluar apoptosis therefore may represent useful biomarkers for efficacious prevention. This *in vitro* model may provide a useful system for understanding the molecular mechanisms underlying normal and neoplastic epithelial cell proliferation.

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ESTRADIOL METABOLISM: AN ENDOCRINE BIOMARKER FOR MODULATION OF HUMAN MAMMARY CARCINOGENESIS

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ABSTRACT:

The natural estrogen 17β-estradiol (E_2) has a profound influence on proliferation and neoplastic transformation of mammary epithelium. The role of cellular metabolism of E_2 in mammary carcinogenesis, however, remains to be elucidated. Explant culture and cell culture models developed from non-cancerous human mammary tissue were utilized to examine i.) modulation of E_2 metabolism in response to treatment with prototype rodent mammary carcinogens and ii.) ability of the naturally-occurring phytochemical indole-3-carbinol (I3C) to influence E_2 metabolism and regulate aberrant proliferation. In **the two** models, treatment with the chemical carcinogens 7,12-dimethyl benz(a)anthracene (DMBA) and benzo(α)pyrene (BP) **altered the metabolism of E_2** as determined from the radiometric (tritium release) and gas chromatography-mass spectrometry (GC-MS) assays. **This alteration in E_2 metabolism** was accompanied by aberrant proliferation and abrogation of apoptosis as determined by **the extent of** replicative DNA synthesis, S phase fraction and Sub G_0 (apoptotic) peak. Exposure of carcinogen-initiated cultures to I3C resulted in induction of C2-hydroxylation of E_2 and of apoptosis and down-regulation of hyperproliferation. Determination of altered cellular metabolism of E_2 in response to initiators and modulators of carcinogenesis and evaluation of cell cycle related markers for proliferation and apoptosis may provide a mechanism-oriented approach to validate E_2 metabolism as an endocrine biomarker for induction and prevention of human mammary carcinogenesis.

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Inhibition of Proliferation and Modulation of Estradiol Metabolism: Novel Mechanisms for Breast Cancer Prevention By The Phytochemical Indole-3-Carbinol

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Running Title: Prevention of Mammary Carcinogenesis by Indole-3-Carbinol

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Abstract

Aberrant proliferation is an early-occurring intermediate event in carcinogenesis whose inhibition may represent preventive intervention. Indole-3-carbinol (I3C), a glucosinolate metabolite from cruciferous vegetables inhibits organ site carcinogenesis in rodent models. Clinically relevant biochemical and cellular mechanisms for the anticarcinogenic effects of I3C, however, remain unclear. Experiments were conducted on reduction mammoplasty derived 184-B5 cells initiated with chemical carcinogen (184-B5/BP) or with oncogene (184-B5/HER), and on mammary carcinoma derived MDA-MD-231 cells to examine whether i.) I3C inhibits aberrant proliferation in initiated and transformed cells and ii.) inhibition of aberrant proliferation is associated with altered cell cycle progression, estradiol (E_2) metabolism and apoptosis. Aberrant proliferation in 184-B5/BP, 184-B5/HER and MDA-MB-231 cells was evident by a 55%-67% decrease in the ratio of quiescent ($Q=G_0$) to proliferative ($P=S+M$) phase of the cell cycle, a 72-90% decrease in apoptosis and a 76-106% increase in anchorage-dependent growth. These cells also exhibited a 88-90% decrease in the ratio of C2-and C16α-hydroxylation products of E_2. Treatment of 184-B5/BP, 184-B5/HER and MDA-MB-231 cells to cytostatic dose of 50 μM I3C resulted in an 137-210% increase in Q/P ratio, a 4-18 fold increase in E_2 metabolite ratio, a 2 fold increase in cellular apoptosis, and a 54-61% inhibition of growth. The preventive efficacy of I3C on human mammary carcinogenesis may, in part, be due to its ability to regulate cell cycle progression, increase the formation of antiproliferative E_2 metabolite and induce cellular apoptosis.

X. ACRONYMS AND SYMBOLS

2-OHE_1	2-hydroxyestrone
16α-OHE_1	16α-hydroxyestrone
AD-CFE	Anchorage-dependent colony forming efficiency
AI-CFE	Anchorage-independent colony forming efficiency
ANOVA	Analysis of variance
BP	Benzo(α)pyrene
cdk	cyclin dependent kinase
E_2	17β-Estradiol
DMBA	7,12-dimethylbenz(a)anthracene
DMSO	Dimethylsulfoxide
EGCG	(-) epigallocatechin gallate
EPA	Eicosapentaenoic acid
FACS	Fluorescence assisted cell sorting
FITC	Fluorescein isothiocyanate
GC-MS	Gas chromatography-Mass spectrometry
GEN	Genistein
GTP	Guanine triphosphate
I3C	Indole-3-carbinol
PCNA	Proliferating cell nuclear antigen
PDT	Population doubling time
PI	Propidium iodide
RP-HPLC	Reverse phase-high pressure liquid chromatography
SEPB	Surrogate endpoint biomarkers

TCA	Trichloroacetic acid
TDLU	Terminal duct lobular unit

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